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FIBRINOGEN DETERMINATIONS AND AFIBRINOGENEMIA*

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Fibrinogen is a circulating labile globulin formed in the liver. Its conversion to fibrin in the presence of thrombin is the end product of the clotting process. In plasma, the normal is 300 mg.% to 500 mg.%. It is increased during inflammatory or infectious conditions, menstruation, and pregnancy.

Fibrinogen deficiency is found as hereditary or congenital hypo- or afibrinogenemia; in association with obstetrical conditions; extensive surgical procedures; and certain miscellaneous disorders. It is thought that the cause is the entrance of placental and pulmonary tissue extracts, amniotic fluid, or other thromboplastic substances into the circulation, thereby producing a diffuse precipitation of most of the plasma fibrinogen leaving the blood, thereafter, incoagulable,[12] or, the presence of fibrinolytic agents in the blood stream, such as plasmin, which prevents the formation of an adequate fibrin clot.[15,23]

Plasmin was first described by Dastre[3] in 1893. He called it fibrinolysin. Plasmin is becoming the accepted term since constituents of the blood other than fibrinogen are lysed by this enzyme.

In rare instances, patients are born with a decrease or a complete lack of fibrinogen although the other components of the blood coagulation mechanism are normal.[10] According to the literature,[4,8,14,16,18] the majority of clinical cases of hypofibrinogenemia are found in association with obstetrical conditions, such as, premature separation of the placenta, abortion, retroplacental hematoma, dead fetus, and toxemia. The low over-all incidence and mortality rates of premature separation of the placenta tends to obscure the fact that the number of deaths is very high in severe cases.

According to Miller,[10] at the time of and shortly following major operations, the patient who has previously had no evidence of hemorrhagic abnormality may begin to bleed profusely. This condition is associated with systemic intravascular coagulation (defibrination) caused by tissue thromboplastic substances. The phenomenon is most frequently encountered in extensive operations on the lung, pancreas, and prostate.

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Reduced fibrinogen levels have also been noted in pernicious anemia, myelogenous leukemia, erythremia, widespread destruction of the bone marrow as a result of metastasis from prostatic carcinoma, and other bone marrow disorders, scurvy, pellagra, extensive burns, infectious diseases, malignancies, and in impaired absorption from the gastrointestinal tract.[24] The cause of the fibrinogen deficiency of these states is unknown. Only in rare cases is afibrinogenemia congenital.

There are various methods of quantitative determination of fibrinogen. Some of these are: gravimetric,[21] nitrogen,[2] colorimetric determinations,[25] determination or turbidity of flocculated fibrinogen or the plasma clot,[5,9] refractometric,[17] viscosimetric,[13] electrophoretic,[7] and determination of the highest plasma dilution at which a clot can be obtained.[19]

Gravimetric methods have not attained wide use since only about 2.8 mg. of fibrin is obtained from 1.0 ml. of plasma.

Brand and Associates[1] determined the nitrogen content of fibrin obtained from a fraction designated as Cohn's fraction I of human plasma. They stated that this fraction contained 87% clottable fibrinogen of which the nitrogen content was 16.9%. Fibrinogen determination by nitrogen content has been proven to be an accurate method, but is time consuming and does not answer the need for a rapid test in afibrinogenemia.

Wu[25] was the first to apply a colorimetric procedure for determining fibrinogen. Other colorimetric procedures have since been devised, but the time element involved in determining these tests would make them impractical.

Gerok[6] published his work in 1952 in which he flocculated fibrinogen by dilution with a buffered solution of 8 M sodium chloride. The following year a method by Parentjev[11] based on this procedure was published. It was later adapted to the Coleman Junior Spectrophotometer by A. H. Fowell.[5]

Losner and Associates[9] perfected a method of determining the turbidity of a plasma clot by adding thromboplastin to plasma and determining the optical density in a spectrophotometer at 500 mu, then adding calcium chloride to the specimen and again determining the optical density when it had reached a constant value. The difference between the two values was directly proportional to the concentration of fibrinogen. This technique was found to be fairly accurate, but had to be strictly standardized.

The fibrinogen concentration of blood may be calculated from the difference in refraction index between plasma and serum.[17] According to Starlinger,[20] this method is unreliable.

The viscosimetric determination[13] is based on the fact that the fibrinogen concentration is directly proportional to the difference in rate of flow of plasma and serum. This test requires 20-25 ml. of blood, the temperature must be carefully controlled, and false high results are obtained in patients with multiple myeloma.

Determination of fibrinogen by free electrophoresis is not practical for diagnosis of hypofibrinogenemia since 6 to 18 hours are required for fractionation of the proteins.

Schneider[19] added thrombin to a serial dilution of plasma and determined the highest dilution of plasma at which a clot could be obtained.

He recognized that the method was inexact, but suggested that, since it was rapid and simple, that it might be useful "as a guide to therapy of intravascular defibrination."

Methods

At times it is difficult to obtain rapid fibrinogen levels by conventional methods, which are usually time consuming. It has been our experience that the techniques of Parentjev[5] and Weiner[22] are advantageous from the standpoint of time and clinical reliability for diagnosis and treatment of afibrinogenemia.

Parentjev's test is a six-step procedure:

1. Five ml. of blood is collected in 0.5 ml. of 4% Sodium Citrate.
2. Plasma is obtained by centrifuging for 10 minutes.
3. Set up blank by using 1 ml. plasma plus 9 ml. of normal saline. Set up sample tube using 1 ml. plasma plus 9 ml. of Parentjev ammonium sulfate reagent.
4. Set the Coleman Junior (Model 6A) spectrophotometer at 100% transmittance and wave length of 510 mu with the blank.
5. The optical density of the sample is obtained **exactly 3 minutes** after its formation. If coagulation has occurred, the sample is given a vigorous shake just before the reading is taken.
6. The fibrinogen concentration of the sample is given in grams per 100 ml. plasma by the equation

$$\% \text{ fibrinogen} = \frac{\text{O.D.} + 0.019}{0.509}$$

where O.D. = optical density.

This test can be completed within 15 minutes after withdrawal of the blood.

In Weiner's method, 5 ml. of blood is drawn in a clean test tube and observed for clot formation at 37° C. Hemostasis is considered inadequate if the clot, when incubated at 37° C., dissolves within an hour. When the fibrinogen level is in the range of the minimal values for hemostasis (100 to 150 mg.%), an unstable clot may form. An unstable clot may also form in the presence of plasmin, or as a combined effect of both of these factors. An unstable clot, regardless of cause, signifies that clinically there is ineffective hemostasis.

Results

The following case reports will point out the value of these technics:

CASE #1: M. G., a 35 year old white female, 6th pregnancy, was admitted to the hospital as a result of an incomplete abortion on January 14, 1956. Previous to this, the patient had a miscarriage in 1951 and an incomplete abortion in 1955 with severe bleeding which ceased after the uterus was curetted. This patient entered the hospital again in 1956 with constant vaginal bleeding and her blood pressure dropped to 76/42. A transfusion was started at this time and blood was drawn for fibrinogen levels after the patient had received approximately 400 ml. of whole blood. A fibrinogen value of 145 mg.% was obtained with Parentjev's method and Weiner's clot observation test suggested hypofibrinogenemia which was corroborated with the result of Parentjev's test.

The patient was returned to surgery and a second curettage was performed. Small pieces of placental tissue were removed and the uterus was packed. This patient received a total of 2 units of blood and 3 gms. of fibrinogen. Five days after the patient was admitted, the fibrinogen content was 370 mg.% and the clot observation test was normal.

Final Diagnosis: 1. Incomplete Abortion.
2. Hypofibrinogenemia.

The patient recovered and was discharged.

CASE #2: C. S., a 24 year old white female, first pregnancy, near term, entered the hospital as a result of a premature separation of the placenta. The patient had vaginal bleeding on entry to the hospital and the bleeding increased after delivery of a dead fetus. Her blood pressure dropped to 70/40. A whole blood transfusion was started and blood was drawn for observation of the clot which appeared to be normal. After the patient had received 6 units of blood, a fibrinogen level of 195 mg.% was obtained. This patient was given three more units of whole blood and 2 gms. of fibrinogen. Subsequent to this therapy, a fibrinogen level of 500 mg.% was obtained. Twenty-four hours later, the fibrinogen level was 450 mg.%

Final Diagnosis: Premature Separation of Placenta and Associated Hypofibrinogenemia.

The patient recovered and was discharged ten days after admission.

CASE #3: A 21 year old white female was admitted for her third cesarean section. She was considered to be in excellent physical condition. There was no remarkable incidence during surgery. The blood pressure gradually dropped from 124/60 to 84/70 in 18 hours. The patient was explored and a slipped suture on a vessel on the fallopian tube was found and ligated. There was no pulse or blood pressure during surgery. Following surgery, the blood pressure was 74/40 and one half hour later the blood pressure was zero and remained so for one hour. After this, the blood pressure varied between 0 and 132/80. She was again explored twenty-four hours after her cesarean section. A hysterectomy was done in an atempt to control the hemorrhage. The patient expired on the operating table.

One fibrinogen determination was done by the Parentjev's method and was 96 mg.%. Two grams of fibrinogen were given at that time. The patient was also given a total of 24 transfusions of whole blood from the time of admission to the time of expiration.

A commercial form of fibrinogen was given along with the whole blood in all three of these cases.

Discussion

The author believes that the fibrinogen determinations that were performed on these patients were an aid to the physician in establishing a diagnosis of hypofibrinogenemia. Physicians and medical technologists should be conscious of the fact that rapid and reliable tests for a fibrinogenemia can be performed in the average laboratory for its diagnosis and treatment.

Parentjev's test should be used primarily for diagnosis of afibrinogenemia in severe cases of premature separation of the placenta and

other conditions in which a rapid diagnosis is necessary. Weiner's clot observation test run in conjunction with Parentjev's test will suggest the presence of afibrinogenemia plus the presence of plasmin in cases in which the time element is not too critical.

Summary

Afibrinogenemia is a condition in which there is a complete absence or decrease of fibrinogen. Its true etiology is not completely understood, but satisfactory methods for the determination of fibrinogen deficiency are available. Rapid diagnosis of afibrinogenemia is of importance in obstetrical cases, extensive surgical cases, and certain other conditions.

Parentjev and Weiner tests are highly recommended from personal experience in the laboratory.

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ANNOUNCEMENTS

Based upon a resolution introduced by the Society's Committee on Legislation before the ASMT House of Delegates in annual convention at Milwaukee, Wisconsin in June 1958, the Board asserts, "The profession of Medical Technology places patient care above selfish interest. Any attempt collectively to negotiate compensation or conditions of employment wherein the physical or mental well-being of the patient may become jeopardized, is contrary to ASMT's concept of professional deportment. The Registered Medical Technologist is by education and specialized training dedicated to uphold rigid standards of duty and performance. In order to maintain such standards, the Society urges its members to stand for maintenance of professional individuality."

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The Mary Francis Gridley Scholarship and/or Loan Fund of the D.C. Society of Medical Technologists announces a $250 scholarship for a three month training program in specialized histological techniques, to be given at the Armed Forces Institute of Pathology, Washington, D.C., beginning in July of 1959.

This scholarship is to help defray living expenses during the three month period, as there are no fees charged for the training course.

Applications may be submitted by any person who is (1) certified by the Board of Registry of the American Society of Clinical Pathologists as a medical technologist or in histologic technic; (2) presently working in the field of histology.

Applications should be received not later than April 15, 1959, and should be addressed to:

Mrs. Martha F. Lanza, MT (ASCP), Chairman
The Mary Francis Gridley Scholarship and/or Loan Fund
Suite 1000—915 19th Street, N.W.
Washington 6, D.C.

USE OF MEDICAL HISTORIES BY THE MYCOLOGY TECHNOLOGIST—A REVIEW*

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World War II experiences stimulated improved technics for the diagnosis of fungus disease by culture, animal inoculation, histopathology, serology, and skin testing. These technics made it possible to study most cases of systemic mycosis and to provide specialized training for the medical technologist within the hospital.

Mounting interest and improved technics have pointed up two facts. First, deep and systematic mycoses can no longer be considered rare diseases. Coccidioidomycosis has been found as an endemic disease over a large part of the southwestern United States.[1] Histoplasmosis was regarded as an obscure tropical disorder a few years ago. It has now been shown[1,5] that 50 to 75% of the people of the central areas of the United States contact a benign form of this disease. Schwarz[6] states that it is more common than tuberculosis in large areas of the United States. Second, is the increasing frequency of fungus infections as an important and sometimes lethal complication of other primary diseases, particularly in cases treated with antibiotics, anti-folic compounds, cortisone, ACTH, and various other compounds. (These complications will comprise Part II of this paper.)

The purpose of this paper is to present a review of pertinent cases I have encountered over an eight year period. These will be presented in three groups of related cases that illustrate certain problems of diagnosis. The case histories of fourteen cases will be omitted from this review as they were routine in nature and presented no unusual problems of diagnosis. They are listed solely for statistical purposes as follows: 2 pulmonary, 2 abdominal, and 5 cervicofacial actinimycosis; 4 pulmonary coccidioidomycosis, and 1 subcutaneous blastomycosis. The fact that 9 cases of actinomycosis in its various manifestations were encountered reflects its endogenous nature and the universal distribution of this organism. The cases of coccidioidomycosis and blastomycosis were in recently discharged troops who had been stationed in endemic areas.

Part I

Group 1. This group of cases, received from other hospitals for diagnosis and/or treatment, may be said to represent laboratory failures and inadequacies; two were believed to be incorrectly diagnosed because of equivocal finding and two were undiagnosed.

Case (1). A 33-year-old white farmer was admitted to this hospital December 5, 1951, because of hemoptysis. He had been hospitalized with pneumonia from December, 1942, to February, 1943, while in the Army. In 1945, following a blow to the chest, he had a sudden hemoptysis, was hospitalized 5 to 6 weeks and treated with penicillin and a sulfonamide. He had repeated hemoptysis and was discharged from the Army for "Actinomycosis of the lungs." In April, 1947, he developed an itchy, raised, erythematous, weeping rash over his left hand and fingers, which recurred intermittently despite sulfonamide therapy.

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On admission to this hospital the rash was present and X-rays revealed multiple bilateral apical cavities with a strong suggestion of bronchial communication. At the time cultures were made for actinomycosis, other fungus cultures were made on Sabauroud's (25° C) and Thompson's (37° C). In approximately eight days cultures on Thompson's medium were showing small, smooth yeast-like colonies and large, slightly wrinkled yeast-like colonies. By both culture and animal inoculation these organisms were identified as *Histoplasma capsulatum* and *Blastomyces dermatitidis*. Cultures of the biopsy of the wrist lesion were negative for fungi although the appearance of the lesion strongly suggested B. *dermatitidis* as the etiologic agent. Numerous cultures of the sputum for actinomycosis were negative.

On February 2, 1952, a right upper lobe lobectomy was done. The infected lung tissue was negative for actinomycotic granules or organisms; however, cultures were positive for *H. capsulatum* and *B. dermatitidis*. In histolological sections no fungus organisms were found in the hematoxylin and eosin stain in the wall of the lesion. There were blastomyces in the exudate covering one of the ulcerated areas, and three large phagocytes contained small, well encapsulatd histoplasma organisms. Periodic acid-Schiff stains demonstrated both organisms although they were rare.

Since cultures remained positive for both *B. dermatitidis* and *H. capsulatum*, a left upper lobe resection was considered and the question of treatment with stilbamidine was entertained. However, on March 3, 1952, the patient left the hospital against medical advice and failed to return.

The chart of this patient was later obtained from another hospital, where he had been admitted in June, 1954, for a possible myocardial infarction. At that time his left thumb was again infected and his biopsy at this time revealed *B. dermatitidis*. He was treated with two courses of 2-hydroxy-stilbamidine without significant changes in the lung cavities although the thumb lesion improved considerably. Three sputum cultures were said to be negative for fungus. In 1956 he was still living although his ultimate prognosis was thought to be poor.

Since most lung infections with actinomycosis are in the lower lobes, a careful examination of the patient's old chart was made. The diagnosis "actinomycosis of the lungs" was based on laboratory slips reporting "actinomycosis" and "*A. bovis*" seen on smears. There were no reports of positive anaerobic cultures or finding actinomycotic granules in sputum. A short notation on a consultation sheet was of interest in that the consultant stated that he believed the organisms seen in sputum smears to be the normal inhabitants of the mouth and did not represent an infection with actinomyces.

This case is one of dual infection with *Histoplasma capsulatum* and *Blastomyces dermatitidis*, on which a mistaken diagnosis of "actinomycosis of the lungs" was made, based on sputum smears alone. Although pulmonary actinomycosis is probably oral in origin, according to Garrod[3] the presence of actinomycosis in the sputum or even in bronchial washings does not indicate infection is present. The diagnosis *must always be based on pus*, and in sputum actinomycotic granules should be present.

Case (2). A 56-year-old, white, hospital laundry worker was admitted to this hospital for the first time on December 30, 1954, with carcinoma of the right upper lobe.

In July 1953 at another hospital he was diagnosed as having advanced emphysema. On December 8, 1953, his X-rays were again read as showing advanced emphysema and an irregular density occupying much of the right upper lobe extending from the hilum upwards into the medial portion of the chest with the sugggestion of a small cavity. Many cultures for *Mycobacterium tuberculosis* were negative but on December 23, 1953, fungi were reported in sputum with the expressed opinion that *Cryptococcus neoformans* was present. *C. neoformans* was also reported in cultures of the bronchial secretions. During the next seven months cryptococcus was reported consistently in sputa. Attention was completely turned to cryptococcus as the primary disease and he was treated with 2-hydroxy-stilbamidine injections and finally with atabrine with no improvement of his condition. Finally on December 1, a bronchoscopy showed a tumor mass arising 1 cm. beyond the origin of the right stem bronchus. Biopsy on December 13, was read as anaplastic squamous cell carcinoma of the bronchus. He was transferred to this hospital for irradiation on December 30, 1954. His deterioration was very rapid and he expired January 9, 1955.

Cultures before death grew a few colonies of *Candida* species. No growth of cryptococcus was obtained on any cultures. Cultures taken at post mortem of pericardial fluid, lung tissue and leptomeninges were negative for bacterial pathogens and fungi. Microscopically there was no evidence of localized or systemic fungus disease.

Review of the patient's chart indicated that the diagnosis of *C. neoformans* infection was based entirely on yeast-like forms being seen in direct smears of sputa and bronchial secretions. There were no special studies nor animal inoculations noted. Correspondence with the pathologist and the attending physician indicated that there was no proof that *C. neoformans* ever existed in this man's lungs. Post mortem findings indicated that surgery was possible many months after tumor growth began; everything suggested that a lobectomy could have been accomplished. Unfortunately, since attention was directed to cryptococcosis as the primary disease, a vigorous cytological survey was never attempted in this case.

That *C. neoformans* can involve the lungs is well known; just how frequently it does is still speculative. However, the identification of yeast-like organisms from sputum as *C. neoformans* should be made with great caution. They must be differentiated from other yeast-like organisms by biochemical and physiological tests, show ability to grow at 37° C, and animal pathogenicity must be determined by intracerebral inoculation of the organisms into mice.

Case (3). A 63-year-old white male was admitted to this hospital July 5, 1951, with the chief complaint of a mass in the left breast of three months duration. The patient had been perfectly well until March 1, 1951, at which time a chest cold had occurred, productive of white sputum. A few days after its onset, a tender, reddened, sore mass developed in the upper quadrant of his abdomen. The mass did not respond to penicillin treatment and ten days later the mass was excised at another hospital. Pathological diagnosis was "acute chronic myositis." Soon another mass developed just above the right iliac crest which slowly increased in size and another mass similar to the one on the iliac crest developed on the left thigh. These masses were incised and drained

and thick purulent material was obtained. The patient was treated with chloromycetin, terramycin, gantrisin, and penicillin. Drainage from the various wounds improved and the patient was discharged April 20 with a diagnosis of multiple abcesses, etiology unknown.

In May a mass in the left breast developed in size but improved in June. In July the mass was again enlarged and painful with a mass in the left axilla so the patient was referred to this hospital. Three days after admission the mass was excised and drained, and 100 cc of bloody, purulent material was sent to the bacteriology laboratory for examination and culture. A Gram's stain of the material revealed many Gram positive filamentous branching organisms approximately 1 mu in diameter. Acid fast stains by the Ziehl-Neelson technic showed these organisms to be partially acid fast. Cultures grew well aerobically on various media. The organism was highly virulent for guinea pigs and classified as *Nocardia asteroides*, an aerobic actinomycete. Sensitivity studies showed this organism to be highly resistant.

Skin graft was started August 7, but due to remaining underlying infection with Nocardia, a simple mastectomy was performed. During this period the patient received both chloromycetin and aureomycin. On October 1, 1951, a successful skin grafting was performed. On October 15, 1951, the patient was discharged with a diagnosis of multiple subcutaneous abscesses due to *Nocardia asteroides*.

This case is a striking example of inadequate bacteriological studies. In reviewing the history supplied by the medical records librarian at the first hospital, laboratory coverage appeared to be adequate except that *no bacteriology had been performed on the material obtained from the multiple abscesses*. Whether this was due to a breakdown of communications between the operating room and laboratory cannot be said. The fact remains that a simple Gram stain would have established an actinomycete as the etiologic agent producing the multiple abscesses.

Case (4). A 33-year-old mechanical engineering draftsman was admitted to this hospital May 19, 1950, because of a mass in the neck. His admission diagnosis was chronic carbuncle with regional adenitis and marked resistance to antibiotics. The patient was in good health until five weeks before when he noted a pimple on the back of his neck on the left side. The lesion became larger and undurated and was treated variously with penicillin packs and ointments by his dermatologist without improvement. A biopsy by the dermatologist and again at another hospital were reported as showing infected granulation tissue without evidence of specific granuloma. Further surgery was recommended to remove the mass and enlarged lymph node but the patient declined and sought hospitalization at this hospital.

On May 26 excision of the granuloma of the neck was carried out. On June 6, the abscess at the base of the neck was removed. The pathological diagnosis was granuloma, etiology unknown. Periodic acid-Schiff stains were negative. Both biopsies were negative for tuberculosis. However, after approximately five days fungus cultures were beginning to show flat membranous colonies at 25° C which later became white and velvety. The organism was identified as *Blastomyces dermatitidis* by cultures

and by animal inoculation. The slides from his previous biopsy were obtained from the other hospital and carefully reviewed. In one of the small abscess areas two large yeast-like cells were seen. One was budding off a daughter cell. This biopsy was then diagnosed as blastomyce of the skin.

This case again illustrates the difficulty in diagnosing a fungus infection, particularly when the etiologic agent is not suspected. Wells[6] states that fungus infections must be considered in the presence of any obscure granuloma which fail to resolve under ordinary antibacterial treatment. That the organisms were overlooked in the hematoxylin and eosin stains of the earlier biopsy is understandable as the organisms are often difficult to find in micro abscesses. However, Conant[1] points out that periodic acid-Schiff stains should always be done on skin biopsies where abscess and chronic granulation are found. There were no reports to indicate that fungus cultures were done on the earlier biopsies.

Group 2. In this group of cases the histology technologist performs a valuable service and the importance of special fungus stains is well demonstrated. There are three cases in this group: two represent so-called "coin" lesions, one a case of Addison's disease.

Case (1). A 35 year old white male was admitted to this hospital on February 14, 1955. He was referred by his doctor because of a shadow in his left lung found by routine X-ray examination in November, 1953. His system review was essentially negative. There was no history of pneumonia or other respiratory difficulties. His chief complaint was chest pain and a slight morning cough. There was no weight loss nor anorexia but he felt less energetic. First and second strength tuberculin tests were negative; Histoplasmin skin test was negative, but the Coccidioidin skin test was strongly positive. His X-ray findings were that of "coin" type lesion in the left midlung field. No definite calcification was seen within the lesion. The lesion showed no change from November, 1953, but tuberculoma and carcinoma were to be ruled out.

A wedge resection of the lung was performed. The tissue measured 3 cm. x 1½ cm. and contained a nodule 1 cm. x 1 cm. Cross section revealed an unsloughed, whitish, firm lesion. Microscopically the specimen consisted of a necrotic encapsulated focus within lung tissue. Around the periphery of the necrotic focus there were many spherical bodies which had a homogenous ground glass appearance and sharp borders. There were also several partially ruptured spherules that contained endospores characteristic of *Coccidioides immitis*. In some sections a few endospores were showing signs of germinating and rare short hyphae were seen. Cultures and animal inoculation of the tissue were negative for fungus and Mycobacterium.

Case (2). A 37 year old male butcher entered this hospital June 1, 1955. He had been referred by his physician because his routine X-ray showed a pulmonary shadow. His system review was relatively normal. His first and second strength tuberculin tests were negative, the Histoplasmin skin test was strongly positive (4 plus), the Coccidioidin skin test was read as 1 plus. His tuberculosis and fungus cultures were nega-

tive. The X-ray impressions were (1) small cystic lesion, right upper lobe, and (2) small inflammatory, vascular or neoplastic lesion in the right upper lobe. Since neoplasm could not be ruled out the patient was brought to surgery.

Specimens received by the laboratory consisted of three parts. The first, from the right upper lobe, was a 6 mm diameter gray white nodule consisting of firm material. The second specimen from the right upper lobe was a small, firm, yellow flecked module about 2 mm. in diameter, which appeared to be partially covered by glistening pleura. The third specimen was a 2 x 2 cm ovoid piece of pulmonary tissue covered by yellowish-white thick pleura with scattered small, satellite lesions studding its surface. Section showed a central, firm, yellow amorphous mass about 3 mm. in diameter containing some anthracotic pigment.

Microscopically the lesions were similar by hematoxylin and eosin stain. Each was granulomatous with amorphous eosinophilic centers and anthracotic flecks. No details suggesting the etiology of the granulomata was given by hematoxylin and eosin stain. Periodic acid-Schiff, Gridley, and acid fast stain by Ziehl-Neelson method were done. These special fungus stains did not reveal the etiologic agent, but the acid fast stains showed patches and nests of acid fast organisms and a tentative diagnosis of tuberculosis was made.

However, in view of the fact that cultures for Mycobacterium tuberculosis remained negative and the histology technologist was experiencing difficulty with her acid fast stains by obtaining false positives on her controls, the current method of acid fast staining was discontinued and Putt's method set up. By this method no acid fast were found in the tissue. Because of the granulomatous nature of the lesion, the chief of pathology then requested that sections be stained by Gomori's methenamine-silver nitrate technic. In these slides many small, round bodies morphologically characteristic of *Histoplasma capsulatum* were found in several foci in the caseous portion of the lesions. All fungus and tuberculosis cultures of the tissue were negative after prolonged incubation.

Case (3). A 65 year old white retired Army major developed symptoms of Addison's disease five years prior to his death May 29, 1954. His chest X-rays were negative and there were no symptoms of tuberculosis.

Autopsy showed death to be due to an arteriosclerotic abdominal aortic aneurysm. Addison's disease was due to caseous destruction of both adrenals without evidence of active tuberculosis elsewhere in the body. Pathological diagnosis in respect to the adrenal glands was recorded as Addison's disease due to bilateral caseous tuberculosis of the adrenals.

At a later date the chief of pathology, who had an occasion to review this case, was impressed by the lack of tuberculosis elsewhere in the body. The adrenal glands were restudied and described as follows: "Both adrenals were moderately enlarged. They were nodular with firm, rounded nodules which were white and firm and apparently tuberculous. Sections through the encapsulated necrotic material revealed its ancient nature with crystal clefts among which were some bluish bodies. These did not stain well with Gridley stain, but were perfectly

characteristic of histoplasmosis with Gomori's methenamine-silver nitrate stain." The etiologic agent causing the destruction of the adrenals was *Histoplasma* rather than tuberculosis.

Discussion: Case 1 is a classic example of the solitary pulmonary nodule as described by Davis et al[2] in their fine article, "The Solitary Pulmonary Nodule." Although Case 2 does not entirely fall into this category, certain features justify its inclusion in a discussion of "coin-like" lesions. In their study the most common single entity has been the granuloma. In their analysis of "reported series" which included over 1000 cases, 41.6% of all cases were granulomas. In an analysis of their own series based on 215 carefully selected cases, 82 (38.1%) were granulomas. Of the 82 granulomas (radiographically non-calcified) 39 (55%) were histoplasmoma, 5 (7%) were coccidioma and only 12 (17%) were tuberculomas.

Their experience with stains reflect the findings exemplified by the three cases reported. These writers[2] concede that in active lesions particularly in the presence of progressive disease, the ordinary hematoxylin and eosin stain is quite satisfactory for diagnosing the deep mycoses. However, in mild, healing or "burnt" out infections fungi are apt to stain poorly if at all by this method and require a special stain such as periodic acid-Schiff, Gridley or methenamine silver nitrate for their demonstration. The advantages of Gomori's methenamine-silver nitrate method as a staining procedure for fungi in tissue sections and smears have been adequately discussed by Grocott.[4] Our experiences have shown this stain to be most important in detecting *Histoplasma* in tissue and is superior to periodic acid-Schiff and Gridley stains in some circumstances.

Cultures and animal inoculations were negative in all three cases described. This compares with the findings of Straub and Schwarz.[5] In their study cultures and animal inoculations were negative in 40 cases showing primary healed complex. In their study of 105 cases dying from other causes, 70 showed healed pulmonary histoplasmosis. They point out that even subclinical histoplasmosis is frequently systemic as indicated by lesions in the adrenals. This is well illustrated by Case 3.

Summary: Since there has not been a proportionate increase in fatal mycotic infections, it is apparent that the rising incidence of fungus diseases is in part a reflection of heightened interest, the development of better methods of diagnosis of early and benign forms and improved staining technics for diagnosis of old, "burnt" out granulomatous lesions.

Four cases are reported that illustrate the inadequacies and pitfalls that a technologist must avoid in making an identification of fungus in material received by the laboratory. Unless findings are unequivocal (culture, animal inoculation, biochemical and physiological studies, special stains) a positive identification should not be made.

Three cases are reported in which old, healed lesions are discussed. These are usually of primary importance only in distinguishing them from carcinoma or tuberculoma. In such lesions the bacteriologist or mycologist can offer little positive assistance in identifying the etiologic agent. On the other hand the histology technologist, with his special

staining methods, frequently gives the pathologist his answer in a row of neatly labeled slides. In order better to assist the pathologist in arriving at a proper diagnosis, much depends on the histologist's attention to such details as the proper preparation and care of stains, the use of controls, and meticulous staining technic.

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Footnote: "Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the results of her own study and do not necessarily reflect the opinion or policy of the Veterans Administration."

PART II

The increasing frequency of fungus infections as important and sometimes lethal complications of other primary disease comprises part two of this paper. The organisms most frequently encountered are *Aspergillus fumigatus and Candida albicans.* In cases such as described in Group 3 careful bacteriologic studies coupled with X-ray findings will frequently determine the etiologic agent before surgery is attempted.

Group 3. This group of three cases represents secondary lung infections with *Aspergillus fumigatus.* A fourth case treated by lobectomy is omitted since it is similar to Case 3.

Case (1). A 37 year old white male social worker was first admitted to this hospital on April 21, 1953 for pulmonary tuberculosis, moderately advanced, active. Starting in February 1953 at another hospital he was treated with streptomycin and isoniazid until February 1955 except for a two months break in January 1954 when a wedge resection of the superior segment of the left lower lobe and an apical posterior segmental resection of the left upper lobe were performed.

The patient's postoperative course was stormy with spikes of fever of 102° to 104° for approximately four weeks, due to aspiration pneumonia in the left upper lobe. Thereafter he had chronic bronchitis and he continued to be febrile. Many sputum cultures were ordered and in February 1954 these consistently showed a good growth of *Aspergillus fumigatus.* Direct examinations of sputa revealed short septate hyphae in moderate numbers. *Aspergillus* was still present in the sputum at the time the patient was discharged on August 12, 1954.

The patient was continued on outpatient status with periodic sputum cultures and X-rays. A planigram of the left chest on September 15, 1954 was read as showing a large cavity-like triangular lesion with the apex at the hilum. Within this lesion there was a smooth homogenous density which appeared to be lying free in the cavity and was thought

to represent inspissated secretions in the necrotic material. In the lateral view there appeared to be a communicating bronchus.

Finally in May 1955 the patient was seen in conference by the consultant in thoracic surgery. He believed the abnormal shadow in the left upper posterior chest to represent an "aspergilloma," probable interpleural, with possible residual bronchial communication. Surgical exploration and excision were strongly recommended.

The patient was readmitted to this hospital on May 31, 1955 and on June 9 was taken to surgery. Pathology at operation consisted of a pleural pocket, extending from the undersurface of the fifth rib in the posterior gutter, filled with grumous, gray-green, dry material. At the bottom of the pleural pocket there were two large bronchial fistulas in direct communication with the pocket. The underlying lung beneath the empyema pocket was palpably normal. Microscopic examination of the material obtained from the pleural pocket disclosed a pure mycelial mat with fresh hemorrhage and much necrosis. Cultures of the material grew *Aspergillus fumigatus*.

Postoperatively the patient did well and has remained well.

Case (2). A 63 year old white male single grinder was admitted to this hospital August 14, 1956 with a diagnosis of pulmonary tuberculosis. Since his admission sputum smears were positive for acid fast bacilli (Gaffky IV) he was started on streptomycin and para-aminosalicylic acid. In July 1957 he had a segmental resection of the left upper lobe. His postoperative course was stormy and he developed tension penumothorax and a wound seratoma.

Due to abnormal X-ray findings, on December 3, sputum was sent to the bacteriology laboratory for fungus and routine cultures. Fungus cultures were rapidly overgrown with *Aspergillus fumigatus*. Grossly his sputum was thick, tenacious and purulent and by direct examination showed moderate septate hyphae. All subsequent specimens were positive for *A. fumigatus*. X-rays on December 6, revealed a cavity or pocket of empyema lying very close to the lateral surface of the pleura. Bronchograms on January 8, 1958 showed communication of the bronchial tree with this space.

He was transferred to the surgical ward and on February 3, 1958 a left exploratory thorocotomy was performed with drainage of the empyema pocket. The cavity was filled with grumous, bloody, gray-green material similar to that obtained in Case (1). Several small fistulas were noted leading from the pleural pocket to the bronchial tree. The pocket had a smooth, intact lining. Microscopic examination of the grumous material revealed a solid mat of hyphae mixed with degenerated cells and red blood cells. Cultures were rapidly overgrown with *A. fumigatus*.

At this writing the patient is still in a surgical ward. Catheters have not been removed from the empyema pocket, but cultures of the drainage were negative for *Aspergillus*.

Case (3). A 31 year old white male thermostat assembler was first admitted to this hospital in July, 1956. For the past two months he was

employed as an assembler at a thermostat company. Prior to that he was employed for six years in a worsted mill in Massachusetts.

Seven months prior to admission he had a four day period of cough with blood streaking which stopped spontaneously, so he did not see a physician. One month prior to admission he had another episode of cough and blood streaked sputum which did not clear up so he visited his plant physician. X-rays taken at that time were read as showing an acorn-like density in the right upper lobe which was interpreted as being a cavity about 2.2 cm in diameter. Further X-rays and planigrams taken after admission were essentially the same and all interpreted as showing right upper lobe cavitary disease, probably of tuberculous etiology.

He was treated for tuberculosis with isoniazid and para-aminosalicylic acid although many sputum and gastric cultures were negative for tuberculosis. There were no fungus cultures requested. His first strength tuberculin test was positive. His X-rays did not change on treatment so he was discharged in September, 1956 and followed in the outpatient clinic.

In February, 1957, his chest plates showed some increase in size of the circular lesion underlying the right clavicle so surgery was advised. On May 13, 1957, a thorocotomy was performed and an apical and posterior segment of the right upper lobe were excised and sent to the laboratory. Within the apical segment was a rounded lesion 2 x 3 cm, which was filled with insipissated, necrotic, greenish material which separated readily from the wall of the lesion. Some of this material was taken for culture. The bronchi passed near the lesion but no communication was demonstrated. Multiple sections through the fixed specimen disclosed a second similar lesion which measured 3 x 3 x 11 mm. This also contained a greenish-yellow, cheesy material. In this, as in the previous lesion, no bronchial communication was established.

Microscopically, the cheesy material was composed of septate hyphae. They did not appear invasive even with Gridley's stain and were found setting directly on the mucous membrane of the cavity. Adjacent bronchioles were plugged with mycelia. Cultures of the cheesy material grew *Aspergillus fumigatus*. There was no tuberculosis in the specimens. Pathological diagnosis was bronchiectasis; chronic bronchitis and pneumonitis, and secondary aspergillosis, right upper lobe.

When last seen in the outpatient clinic in December, 1957, the patient stated he felt better now than he had for the past five years.

Discussion: *Aspergillus fumigatus* is an extremely cosmopolitan mold that grows well at 37° C, a characteristic of most pathogenic fungi. Thom and Raper[2] list many references which cover the occurrence of *A. fumigatus* in lesions of birds and mammals, including man, together with animal experimentation. Primary infections in human beings occasionally occur. Smith Foushee and Norris[4] recently described a case of primary pulmonary aspergillosis treated by lobectomy. They state that their case is the eighth case of localized primary aspergillosis treated by lobectomy reported in the United States to date. Most cases, however, are secondary to some other disease, particularly tuberculosis and bronchiectasis. In the cases discussed here tuberculosis and bronchiectasis were un-

questionably the predisposing cause. None of these cases showed invasion of tissue by mycelia; frequently the fungus mat was seen on the mucous membrane of the cavity.

Since cases (1) and (2) represent post-operative complications, much concern was expressed as to the possible source of contamination with *Aspergillus*. Case (1) probably became infected at the time he developed aspiration pneumonia. As is frequently the case with exogenous mycoses, the portal of entry of *Aspergillus* is the respiratory tract and pulmonary lesions are by far the most common, presumably as a result of aspiration.[6] This may also have been true of case (2) since his post operative course was stormy.

Although bronchopleural fistulas appear to be a fairly common complication in pulmonary resections for tuberculosis, a casual review of some of these reports revealed no case reports of *Aspergillus* complications similar to the two reported here. Undoubtedly some do exist. That this complication represents a real hazard cannot be denied as the patient must undergo the risk of further surgery.

Although there was no proof of tuberculosis in case (3) other than the positive tuberculin test and X-ray findings, his original lesion may have been tuberculosis or focal bronchiectasis. This case resembles the "aspergilloma" mentioned by Davis et al,[1] and the intracavitary "fungus ball" described by Vellios[5] and his associates. It is of some interest that this patient worked in a worsted mill for six years. In Henrici's hand book[3] *Aspergillus* is one of the molds listed that attacks wool and other textiles under adverse conditions. It is conceivable that he became infected through handling contaminated yarn.

I do not believe that the prolonged antibiotic therapy was a predisposing factor to the fungus infections in Group 3. All were in relatively good physical condition. The presence of a mycetoma requires nothing more than a pre-existing pulmonary cavity and exposure to sufficient organisms. They are among the commonest of the solitary pulmonary nodules and are frequently picked up on routine X-ray surveys.

By contrast, it is usually when the patient has become markedly debilitated by leukemias, Hodgkin's disease, etc., that antibiotic and drug therapy appears to be a contributing factor in terminal fungus infections. Our autopsy files over a period of five years contain at least eight cases of terminal infection with *Aspergillus* or *Candida* species.

Case report: This 60 year old white male expired January 25, 1956 and an autopsy was performed on January 26, 1956. He had entered the hospital with hematemesis, developed mesenteric thrombosis and was subjected to a resection of his distal ileum and right colon on an unprepared bowel. Postoperatively he developed a leak in his anastomosis and peritonitis. Following a second operation he developed pulmonary edema and spiked a temperature to 105° (R). Tracheotomy was performed but the patient expired 27 days after admission.

Due to heavy antibiotic therapy (penicillin, streptomycin, chlormycetin, erythromycin, and cortisone for staphylococcal septicemia) he developed terminal system infection with *Candida albicans* and blood cultures taken two days before death grew *C. albicans*. The organs involved

showed little inflammatory response or necrosis associated with the fungus infection, although in the kidneys the individual glomeruli appeared to be obstructed by the fungus. Other glomeruli were partially destroyed and fungi filled the proximal convoluted tubules suggesting focal infarction.

Summary: Three cases are discussed that emphasize the importance of *A. fumigatus* as a secondary invader in pulmonary lesions. Where cavitary lung disease exists and following pulmonary resection complicated by bronchopleural fistula, the abundant and persistent presence of this organism in sputum smears and cultures should not be ignored. In patients already debilitated by disease disseminated *Aspergillus* and *Candida* infections are unquestionably more frequently encountered in the present era of modern therapeutics. This can be attributed to a complex of factors including a substrate of debility (leukemia, etc.), a suitable portal of entry, usually a local lesion in the alimentary or respiratory tract, and an ecologic disturbance brought about by antibiotics, steroids, and other compounds.

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MECHANICAL WASHER FOR ELECTROPHORESIS FILTER PAPER[1]*†

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The removal of background dye and differentiation of the fractionated protein on filter paper used in electrophoretic determinations is greatly facilitated by agitation. The mechanical rocker described below has proved to be very satisfactory and time saving. It was designed primarily for processing strips dyed with Amido-Schwarz B10. Since the dye vehicle is methanol, hand rocking exposes the operator to considerable vapor unless a hood is available. Bromphenol blue stained paper requires several hours or overnight differentiation. By using the mechanical washer the processing is accomplished in thirty minutes.

Description of Apparatus

Power is furnished by a small 110-volt electric motor with gear reduction 400:1 delivering 28 r.p.m. Rotary motion is converted to reciprocating motion by a cam. The profile is shown in Fig. 1. During the period of cam rise the dye or washing solution flows over the paper slowly and returns in a wave motion during the quick return to the period of rest. The pan is spot welded to a ⅛-inch stainless steel shaft which is free to move in the slotted brackets. A cam follower attached to the pan provides the only other moving part. The motor and brackets are anchored to the ¼- by 12- by 21-inch aluminum base plate. The

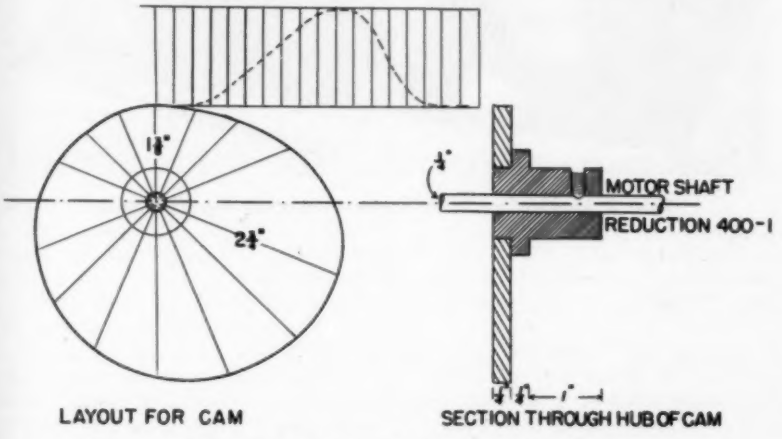

Figure 1. Diagram of mechanical washer for electrophoresis filter paper.

* Received for publication July, 1958.
† A laboratory suggestion.

switch is mounted on the motor base blocks. Fig. 2 shows the assembled machine.

Operation of Washer

Place the dried filter paper strip in the pan and add the selected dye in sufficient amount to cover the paper and produce a wave during operation. Start the motor and run for the required time for staining. Remove the dye by lifting the pan off and decanting. Then add washing solution and continue the process. Make the necessary changes of washing solution and thus complete the differentiation. Hang up the paper to dry.

Credit is given Mr. Charles Chambers, machinist at the laboratory machine shop, for cam design and construction of the apparatus.

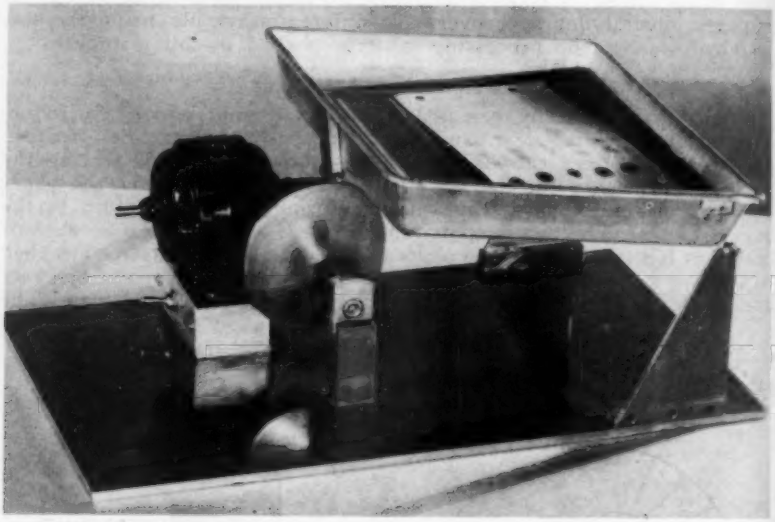

Figure 2. Assembled mechanical washer for electrophoresis filter paper.

VARIATIONS IN ATYPICAL LYMPHOCYTE MORPHOLOGY DUE TO TECHNICAL DIFFERENCES IN BLOOD FILM PREPARATION*

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Since first observed in a case of "atypical mumps" by Curschman[9] in 1906, the pathologic lymphocyte has been the source of much confusion to the physician and the medical technologist. This type of mononuclear cell has been described by many, each giving his own interpretation of its morphology. Workers have agreed that the atypical mononuclear cells seen in increased numbers and diverse morphology in cases of infectious mononucleosis, while not specific for this condition, have been found to occur in a higher percentage of the total count than when seen in other diseases. Workers have not agreed upon a definite classification for these cells which are lymphocytic in origin, although the work of Downey and McKinlay,[5] 1923, has been the classic guide. They grouped the atypical cells into three major divisions with transitional forms existing between each type and the normal lymphocytes. Studying routine and thin blood films on both slides and cover-slips, we noted that the morphology of the atypical cells varied with the thinness of the smear. This made us question the actual existence of types of atypical cells and to what degree the morphologic variation of these was due to discrepancies in the techniques of making and reading blood films.

Downey and McKinlay described the variations in atypical lymphocytes in 1923 and cells have been ambiguously categoried according to this classification since then. Quoting from Downey, Handbook of Hematology,[5] the atypical lymphocytes are classified as follows:

"The cells are not excessively large . . . In spite of the marked lobulation of the nuclei of many of the cells, it is evident that all the cells belong to the lymphocytic series, for it is possible to select a complete series of transitional stages . . ."

Type I: In the nuclear structure "the chromatin forms a coarse network of heavy strands and masses which are not sharply separated from the parachromatin . . . . The nuclei of the larger cells are frequently placed eccentrically and are generally lobulated or indented . . . Many of the smaller cells also have irregular nuclei . . ."

The cytoplasm of Type I cell is very basophilic. "With Wright's stain, the spongioplasm appears as dark blue or slate blue, finely granular, of flaky material with a pale yellowish background of hyaloplasm. Its distribution is such that it gives the cytoplasm a vacuolated, foamy or mottled appearance . . . ."

Type II: In the atypical cell of the Type II cases, "The nucleus is somewhat similar to that of a plasma cell. The chromatin strands are very coarse, and there are several dense, rounded or angular masses of chromatin among them. These cells have a more washed appearance, and the blocking of the chromatin is not so pronounced as in the plasma cell . . .

"The cytoplasm . . . is very different from that of cases belonging to

* 2nd Hematology, SPF Award, 1958. Read before the 26th Annual Convention of ASMT, Milwaukee, Wisconsin, June, 1958.

Type I. It has fewer vacuoles and its spongioplasm has a smoother appearance which does not give the foamy, spongy and stippled effect noted in the cells of Type I. It is generally less basophilic . . . The tendency of the basophilic portion of the cytoplasm is to be arranged in the form of broad bands radiating from the nucleus . . .

Type III: "Type III cases show cells with more leucemic features than those described under Types I and II . . . The cytoplasm is vacuolated and in some cells it is quite basophilic . . .

"Possibly 1% of the lymphoid cells contain a single large vacuole with an azurophile rod, in addition to smaller vacuoles distributed rather evenly throughout the cytoplasm. The larger vacuole is located within an indentation of the nucleus."

Downey found that Type I lymphocytes predominated in 5 out of 9 cases. Other observers have accepted his descriptions and have found most of their cases to be predominant in Type I cells. From our observations we assume the cells described by Downey were observed on blood films made on slides and of a thickness to give good differentiation of the cells but not thin enough to allow erythrocytes to be so widely spaced, that no touching of adjacent cells occurred. For clarity of terms, we will refer to the usual type of preparation which meets all the criteria of a good differential as the thick film; those thin enough to observe each erythrocyte singly will be the thin film; and the ones made by the cover slip method will be called cover slip films.

We have noticed that thick films examined for routine purposes show an increased number of atypical lymphocytes with lobulated nuclei, morphologically resembling those of Type I (Downey). On thin slide and coverslip preparations made at the same time on the same blood specimen, by the same technologist, we find the predominant type of atypical cells is very irregular in shape, is large or larger than those Downey classed as Type II. The cytoplasm near the nucleus is a smooth light grey-blue but deeply basophilic at the outer rim of the cell, especially if it is indented by adjacent erythrocytes. Azurophilic granules may be present. The nucleus is large, round or oval, usually eccentrically placed, and like the cytoplasm is weak structurally and easily pushed out of shape. With Wright's stain, the nucleus has a reddish-purple hue, and the chromatin is diffuse, in no way resembling the nucleus of the Type II cell described by Downey. Bender,[1] found 8% of his patients had cells similar to those we have observed on thin slide and coverslip preparations. He recognized that the characteristics seen on the thin film were all but completely obscured on thick, routine preparations, and he emphasized the importance of the thin film technique for identifying these cells.

We undertook to establish a study of atypical lymphocytes which would confirm our suspicion that technical factors were largely responsible for "types" of atypical lymphocytes. As subjects, we selected cases of infectious mononucleosis where a high percentage of atypical lymphocytes could be anticipated. To date, 22 patients have been studied who met the following criteria for the diagnosis of infectious mononucleosis:

1. Clinical picture classically ascribed to infectious mononucleosis.
2. Change in titer in the heterophile agglutination test. Several sera

collected throughout the course of the patient's illness were tested against the same specimen of sheep cells.[12]

3. An absolute lymphocytosis with 50% or more of the lymphocytes appearing as atypical mononuclear cells.

Initially blood was obtained from each patient for erythrocyte, leukocyte, thrombocyte, and reticulocyte counts, hematocrit, and heterophile agglutination. All blood work was done in duplicate. In addition, polished coverslip preparations, and differential films of two thicknesses were made, one of usual thickness to constitute a good slide and the other very thin throughout the length of the film. Wright's stain was used on all blood films. These studies were repeated several times during the course of the disease. Only the leukocyte count and differential findings will be discussed in this paper.

Leukocyte counts were in duplicate and ranged from 3,500 to 26,000 per cmm. of whole blood, limits which are usual for infectious mononucleosis. Differential counts of 200 cells each were recorded from a thick, a thin, and a coverslip preparation. The morphology of the thrombocytes appeared normal. However, basophilic stippling of an occasional erythrocyte seeemed to be an accompanying feature.

For the study, lymphocytes have been divided into

(a) Normal

(b) Nuclear Types—lobed nucleus with coarse masses of chromatin, and a foamy, basophilic cytoplasm. (Figures 1 and 3).

(c) Cytoplasmic Type—the nucleus, if not distorted by pressure, is large, round, or oval, with diffuse chromatin. Cytoplasm is smooth, grey-blue and is easily distorted into pseudopodia-like projections that indent whenever another cell comes in contact with them. The cytoplasm is light, clear blue near the nucleus

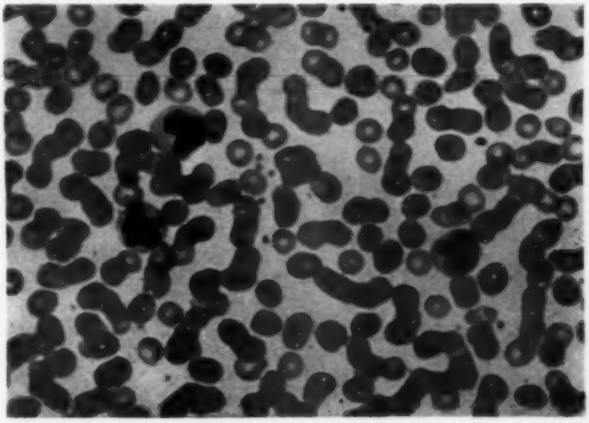

Figure I. Atypical Lymphocytes on thick film. Wright's stain X 450.

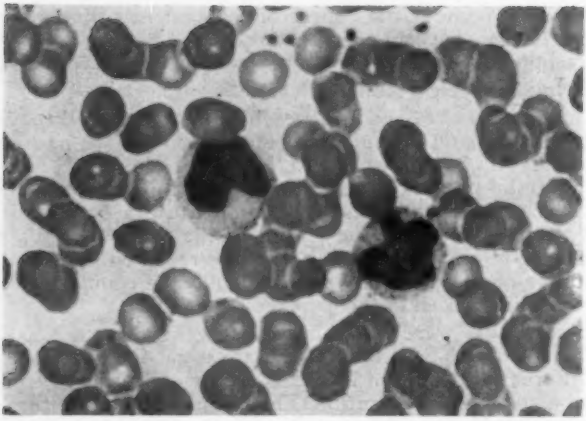

Figure III. Atypical Lymphocytes on thick film. Wright's stain X 910.

and deeply basophilic at the periphery. (Figures 2, 4, 5).
The terms "nuclear" and "cytoplasmic" are inadequate labels for the atypical cell differentiation, but to avoid confusion with the Downey classification, we have employed terms which call attention to the more pathologic feature of the cell. By using these names, we do not wish to imply that in the nuclear class, the cytoplasm is typical, or that the cytoplasmic type has a normal lymphocytic nucleus.

In all cases we have studied in this manner, we have found the cell described in (c) was present in increasingly greater numbers as the film

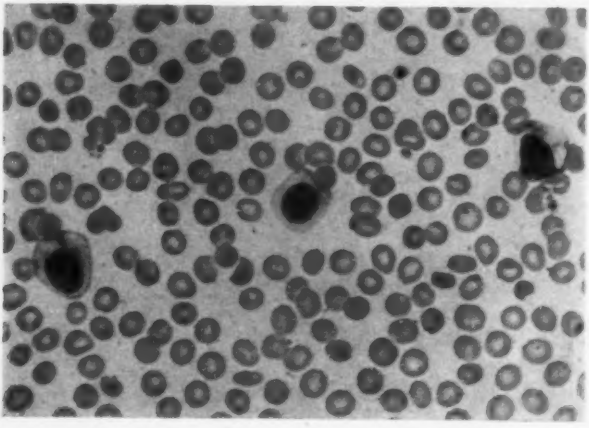

Figure II. Atypical Lymphocytes on coverslip preparation. Wright's stain X 450.

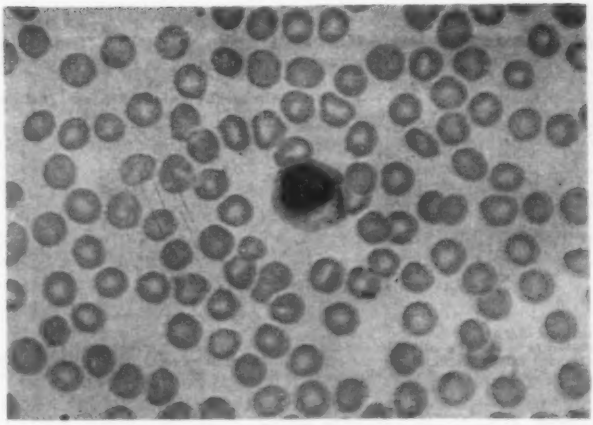

Figure IV. Atypical Lymphocytes on thin film. Wright's stain X 910.

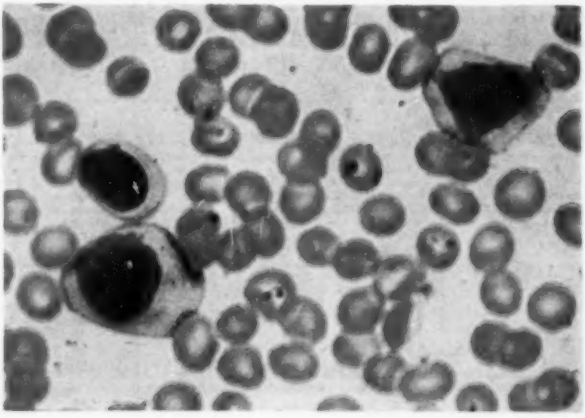

Figure V. Atypical Lymphocytes on coverslip preparation. Wright's stain X 910.

was made thinner. The largest increase in this type of mononuclear cell was seen on the coverslip preparations. In such films, the blood spreads by capillary action and the dispersion of the leukocytes is that of chance distribution. The blood film prepared by spreading a drop of blood on a slide and using another slide as a spreader does not give uniform distribution because the larger cells have a tendency to be pushed to the periphery and to the end of the film. From the cases studied, we have selected at random four illustrations showing the variations in the atypical lymphocyte due to technical variations. The results are shown in the following tables:

Table I

Case I (J.F.) LEUKOCYTE AND DIFFERENTIAL COUNTS AND HETEROPHILE TITERS**

DATE	Day* of illness	Leukocytes per/mm³	Neutrophile		Eosinophils	Basophils	Lymphocytes	Monocytes	Disintegrated***	Heterophile Titer
			Band	Seg.						
2-10-53	11	14,400	8	9	2	0	72	4	5	1:4096
2-12-53	13	19,300	3	21	1	0	68	3	4	
2-14-53	15	23,300	3	17	1	2	70	4	3	
2-16-53	17	22,000	2	15	0	0	76	3	4	
2-18-53	19	20,600	2	15	1	0	75	3	4	1:4096
2-20-53	21	19,300	1	20	0	0	74	2	3	1:4096
2-27-53	28	11,100	0	36	2	0	58	2	2	1:4096
3- 6-53	36	10,500	0	49	1	1	44	4	1	
3-11-53	41	11,700	1	53	2	0	48	5	1	
3-18-53	48	9,900	1	58	0	2	34	3	2	
3-25-53	56	11,300	1	70	0	1	21	5	2	
4-29-53	90	11,100	0	66	0	0	27	3	2	1:16

* Approximate day of illness.
** Differential percentages in all tables are the average of 600 cells, 200 from each film.
*** Disintegrated cells are included in the relative differential as recommended by the Committee for Clarification of the Nomenclature of the Cells and Diseases of the Blood and Blood-Forming Organs, 1956.

Table II

LYMPHOCYTE DIFFERENTIAL STUDY OF CASE I (J.F.)

DATE	Film	Normal	Nuclear	Cytoplasmic	Total Lymphocytes
2-10-53	Thick	43	17	13	73
	Thin	29	12	27	68
	Coverslip	..	..	..	..
2-12-53	Thick	39	20	10	69
	Thin	17	21	30	68
	Coverslip	10	4	50	64
2-14-53	Thick	25	34	7	66
	Thin	9	7	57	73
	Coverslip	5	18	50	73
2-16-53	Thick	26	45	6	77
	Thin	14	6	51	71
	Coverslip	6	10	65	81
2-18-53	Thick	31	34	13	78
	Thin	10	9	54	73
	Coverslip	15	15	45	75
2-20-53	Thick	30	28	15	73
	Thin	22	20	34	76
	Coverslip	4	9	60	73
2-27-53	Thick	42	14	9	65
	Thin	14	13	24	51
	Coverslip	27	12	15	54
3-6-53	Thick	28	9	5	42
	Thin	23	10	8	41
	Coverslip	35	9	5	49
3-11-53	Thick	39	4	1	44
	Thin	25	8	5	38
	Coverslip	25	5	3	33
3-18-53	Thick	25	5	1	31
	Thin	21	1	10	32
	Coverslip	21	2	15	38
3-25-53	Thick	19	1	0	20
	Thin	19	1	1	21
	Coverslip	19	1	1	21
4-29-53	Thick	30	0	0	30
	Thin	29	0	0	29
	Coverslip	23	0	0	23

Case I

J. F., aged 22, male, sophomore medical student, was admitted to the hospital on 2-9-53. He had not been feeling well, had a sore throat, cervical lymphadenopathy, and malaise for ten days preceding admission. On admission, he was acutely ill, febrile, and complained of a severe headache. These classic symptoms was borne out by the hematologic and serologic findings; diagnosis of infectious mononucleosis was made. The course of the disease and convalescence were uneventful. He was afebrile and clinical symptoms had subsided by 2-20-53. He returned to school on 2-27-53. The blood picture and heterophile agglutinins were followed until 4-29-53 when all values had returned to normal. A summary of the leukocyte counts, differentials, and heterophile tests are shown in Tables I and II.

Case II

L. U., aged 19, female, student dental technician, reported to the Student Health Service on 4-21-53. The patient was febrile, had moderate cervical and axillary lymphadenopathy, sore throat, headache, and malaise. In three weeks, the clinical symptoms had subsided. The heterophile titer had dropped. The leukocyte count was normal although the differential still showed approximately 15% atypical lymphocytes. The suspected diagnosis of infectious mononucleosis was confirmed by laboratory data which is recorded in Tables III and IV.

Table III
Case II (L.U.) LEUKOCYTE AND DIFFERENTIAL COUNTS AND HETEROPHILE TITERS

DATE	Day* of Illness	Leukocytes per/mm³	Neutrophile		Eosinophils	Basophils	Lymphocytes	Monocytes	Disintegrated	Heterophile Titer
			Band	Seg.						
4-22-53	10	11,900	4	16	1	1	63	5	6	1:256
4-24-53	12	10,200	2	22	1	0	65	5	5	
5-12-53	30	7,800	0	48	1	1	41	5	4	1:64

* Approximate day of illness.

Table IV
LYMPHOCYTE DIFFERENTIAL STUDY OF CASE II (L.U.)

DATE	Film	Normal	Nuclear	Cytoplasmic	Total Lymphocytes
4-22-53	Thick	46	20	6	72
	Thin	21	11	33	65
	Coverslip	20	11	40	71
4-24-53	Thick	48	17	3	68
	Thin	40	6	17	63
	Coverslip	35	8	21	64
5-12-53	Thick	22	12	6	40
	Thin	22	7	11	40
	Coverslip	30	2	10	42

Case III

H. K., aged 19, male, junior university student, reported to the Student Health Service on 3-6-55. He had been ill for two weeks, unable

to attend classes, and had been treated for a severe sore throat. When seen at the health service, cervical lymph nodes were slightly enlarged and the throat appeared normal, but the patient had periumbilical pains, nausea, anorexia, and fever (101° F). The spleen was slightly palpable. Infectious mononucleosis was suspected. The total leukocyte counts were all within normal limits, but showed a lymphocytosis with many atypical cells. Our initial count was 7,400 WBC/mm,^{3} and the differential study showed 69% lymphocytes, 57 of which were atypical. Serum collected on the same day (3-6-55) had a heterophile titer of 1:56. The patient improved steadily. Seven weeks later, the blood count and differential were normal, and the heterophile titer was zero. Based on the blood film findings and the change in heterophile titer (Tables V and VI), it would appear that this patient had come to our attention after the infection had passed its peak.

Table V
Case III (H.K.) LEUKOCYTE AND DIFFERENTIAL COUNTS AND HETEROPHILE TITERS

DATE	Day* of Illness	Leukocytes per/mm^3	Neutrophile		Eosinophils	Basophils	Lymphocytes	Monocytes	Disintegrated	Heterophile Titer
			Band	Seg.						
3-6-55	15	7,400	1	27	2	0	66	2	2	1:56
3-9-55	18	9,700	0	30	1	0	65	3	1	
3-11-55	20	8,500	0	32	1	1	60	3	3	1:16
3-14-55	23	8,200	0	33	2	0	63	1	1	
3-16-55	24	5,900	0	44	2	1	47	2	4	1:16
3-18-55	26	6,400	0	55	1	1	36	5	2	
3-27-55	35	6,800	0	55	2	0	40	2	1	1:8
5-1-55	70	5,400	0	61	0	0	30	6	3	0

* Approximate day of illness.

Table VI
LYMPHOCYTE DIFFERENTIAL STUDY OF CASE III (H.K.)

DATE	Film	Normal	Nuclear	Cytoplasmic	Total Lymphocytes
3-6-55	Thick	27	29	7	63
	Thin	12	20	37	69
	Coverslip	..	..	..	..
3-9-55	Thick	28	23	13	64
	Thin	24	24	18	66
	Coverslip	17	19	28	64
3-11-55	Thick	33	30	2	65
	Thin	10	3	44	57
	Coverslip	25	6	26	57
3-14-55	Thick	35	10	6	51
	Thin	30	11	22	63
	Coverslip	35	22	14	71
3-16-55	Thick	30	11	5	46
	Thin	32	10	7	49
	Coverslip	20	8	20	48
3-18-55	Thick	25	3	1	29
	Thin	32	3	3	38
	Coverslip	21	1	18	40
3-27-55	Thick	28	5	0	33
	Thin	33	2	4	39
	Coverslip	40	5	5	50
5-1-55	Thick	32	0	0	32
	Thin	32	0	2	34
	Coverslip	31	0	0	31

Case IV

M. M., aged 23, female, housewife, was admitted 5-19-57 with a diagnosis of infectious mononucleosis. Initial blood studies had been made in another laboratory, and had confirmed the diagnosis suspected from her clinical symptoms. The patient was febrile, cervical lymph nodes were enlarged, and she complained of headache, sore throat, nausea, and malaise. She was discharged on 5-23-57 and clinical symptoms had subsided at that time. Laboratory data is recorded in Tables VII and VIII.

Table VII
Case IV (M.M.) LEUKOCYTE AND DIFFERENTIAL COUNTS AND HETEROPHILE TITER

DATE	Day* of illness	Leukocytes per/mm³	Neutrophile		Eosinophils	Basophils	Lymphocytes	Monocytes	Disintegrated	Heterophile Titer
			Band	Seg.						
5-19-57	11	14,100	2	26	0	0	62	6	4	1:256
5-20-57	12	11,900	2	26	0	0	67	4	1	
5-21-57	13	11,000	1	20	0	0	73	3	3	
5-22-57	14	9,100	1	23	0	1	65	6	3	
5-23-57	15	9,100	0	28	0	0	65	5	2	1:128

* Approximate day of illness.

Table VIII
LYMPHOCYTE DIFFERENTIAL STUDY OF CASE IV (M.M.)

DATE	Film	Normal	Nuclear	Cytoplasmic	Total Lymphocytes
5-9-57	Thick	50	16	6	72
	Thin	27	6	31	64
	Coverslip	23	6	28	57
5-20-57	Thick	41	18	9	68
	Thin	24	13	29	66
	Coverslip	39	7	22	68
5-21-57	Thick	55	16	9	80
	Thin	48	5	16	69
	Coverslip	50	7	13	70
5-22-57	Thick	48	14	3	65
	Thin	39	9	20	68
	Coverslip	42	6	17	65
5-23-57	Thick	48	11	5	64
	Thin	45	6	12	63
	Coverslip	50	6	12	68

We feel that the work we have presented shows the need for further study of blood film techniques as a valuable aid in clarifying the conflicting descriptions that have been attributed to what we believe is the same cell type observed under different technical conditions. Examination of the lymphocytic differential tables 2, 4, 6, and 8 reveal a definite increase in the cytoplasmic type cell on the thinner preparations. The increase in this form of lymphocyte is slightly more striking on the coverslip films, although slides of single cell thickness prepared at the same time and from the same patient show an almost equal number, and may exceed the percentage found on the coverslip.

Compared to the results of the thin film, the thick slide shows an increase in both nuclear types and normal lymphocytes, and a definite

decrease in the number of cytoplasmic forms. The increase of normal cells is greater than that of the nuclear type in most instances, and it would appear that the cytoplasmic form often looks like a normal cell on the thick film and this results in a differential count with a false number of normal lymphocytes. This fact could lead to an error in interpretation of the differential count, especially if a single blood study were taken early in the course of the disease before the heterophil antibodies had developed.

Nuclear forms appeared on the routine differential two to three times as frequently as did the cytoplasmic variety of atypical cells; however, the total number of atypical lymphocytes was 15 to 20% less than was found on the films of single cell thickness. It must be borne in mind that regardless of the thinness of the film, leukocyte response in some instances is not sufficiently atypical to warrant the presumptive diagnosis of infectious mononucleosis.[12] Also, blood findings from these cases are easily confused with infectious hepatitis.

Differentiation of atypical lymphocytes with emphasis on technical variations in making of blood films has been studied in 22 cases of infectious mononucleosis presenting classical clinical symptoms. Further studies are indicated, but we feel we have shown that thin preparations, slide or coverslip, should be examined in all cases suspected of infectious mononucleosis. At present, the principal means of diagnosis of the disease is by use of non-specific tests, the heterophile and lymphocyte differentiation. Once the causative agent and its inherent qualities have been established, the whole concept of the disease may change; new specific procedures may replace the present tests, which may be reacting only to a certain strain of the infectious organism, and may be failing to respond to other strains. Meanwhile, the heterophile and morphologic cell differentiation remain the diagnostic tools at our disposal. Such measures must be applied with appreciation of the artifacts which may be introduced, as shown in the work described in this paper and in other reports involving the diagnosis of infectious mononucleosis.

Summary

1. Variations in atypical lymphocytes produced by technical factors were studied throughout the course of the disease in 22 cases of infectious mononucleosis.
2. Classification of atypical lymphocytes was made from coverslip, thin, and thick Wright's stained differential films.
3. Thick films showed the atypical cells with foamy, basophilic cytoplasm, and lobed nuclei with dense chromatin clumps.
4. The thin and coverslip preparations showed an increase in the number of atypical cells which were soft, easily distorted, with cytoplasm deeply basophilic at the periphery and light blue in the perinuclear zone. The nuclei were round or oval and composed of diffuse chromatin.
5. This study showed that technical factors involving thickness of a blood film influence the appearance and number of the atypical lymphocytes.

Acknowledgment

I would like to express my appreciation to Dr. C. J. D. Zarafonetis for his contribution to this paper and for his insistence that the atypical

lymphocyte in Ann Arbor does not look like the atypical lymphocyte in Philadelphia—the statement which led to our study.

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STAINING BLOOD FILMS BY MEANS OF TRAYS AND DISHES*

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Although used in histology for many years, staining trays and dishes (Fig. 1) have not been widely employed in preparing the usual blood film, even though this type of equipment provides many superiorities over the prevalent method of supporting slides on a rack and flooding their surfaces with the various staining materials. Staining by means of trays and dishes (or similar means, such as Fig. 2) is also known

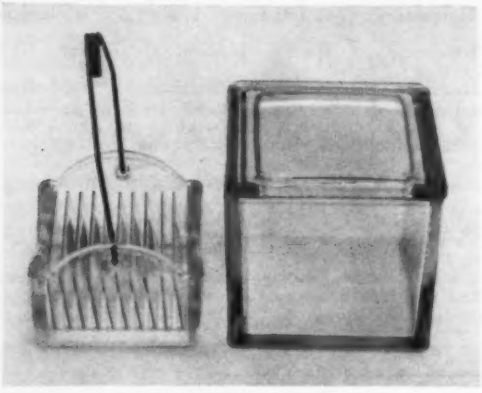

Fig. 1. Staining tray (left) and dish.

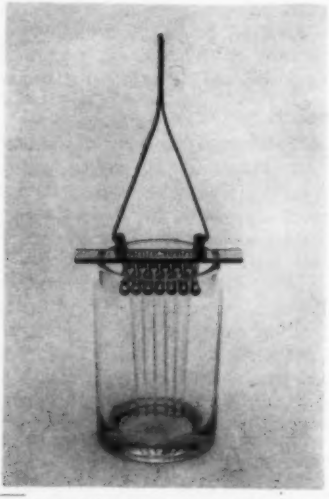

Fig. 2. Another type of equipment for immersion staining, having theoretical advantages over that of Fig. 1. Slides are inserted by pressing toward each other the bars to which the handle is attached. The plastic bar resting on the rim of the jar is not part of the original apparatus.

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by the following terms: the dip technic, the immersion method, mass staining, *en masse* staining, or bulk staining.

The following paragraphs include additional background information concerning the immersion type of procedure, details of one method which was instituted as routine, and observations on its use.

The Romanowsky stains are a commonly used group composed of various combinations of eosin, methylene blue, and methylene blue derivatives produced by its oxidation. These derivatives are of known chemical composition and are termed Azure A, Azure B and Methylene violet. Methylene blue and the derivatives are chemically designated *thiazines*. Often, these same substances are known by other names. Thus, *Azure I* is evidently Azure B or Azure B plus contaminating related dyes. *Azure II* consists of equal parts (w/w) of *Azure I* and methylene blue. Unfortunately, there has been some confusion in the terminology of the *Azure* dyes. Since several dyes are included, Romanowsky stains are also known as trichrome, tetrachrome, or polychrome stains. Derivatives of methylene blue have their color shifted toward red by certain substances being stained. As a result, a structure will appear reddish, even though the dye to which it was subjected is pure blue. This color change, termed *metachromatic* staining, is partly responsible for the wide range of colors obtained. Wright's, Giemsa's, Leishman's, MacNeal's, and Hastings' are among the many varieties of Romanowsky stains, differing slightly and sometimes significantly. For optimum staining of the greatest number of structures, Giemsa's is often used in combination with other stains which are not necessarily true Romanowsky types. The best known of these combinations include Jenner-Giemsa, May-Grünwald-Giemsa, Wright-Giemsa and Leishman-Giemsa.

Intermediate between rack staining and the immersion method is the covering of the dried, unstained film with a 2 cm. by 5 cm. piece of filter paper.[18] With the slide in the open, horizontal position, the staining process is carried out as usual, the paper being removed, however, just before the final wash. Stain may be added at intervals, as required, to keep the paper wet and well-covered. Presenting no barrier to the staining materials, the filter paper maintains more uniform conditions on the surface of the slide, lessening such mishaps as drying or spillage.

A number of workers have constructed devices for bulk staining, even though several types and sizes of holders for slides or coverslips are commercially available. Glassy[8] adapted a metal 3¼ in. by 4 in. staining tray to hold up to sixty coverglass smears. These were placed in Wright's stain for 2-4 min., then buffer for 3-6 min., after which they were air-dried. Mention is made that blood smears on slides can also be stained in the same manner.

A coverglass holder, similar in design to the above, was made of 1 mm. thick polyethylene by Fjeld.[7] Eleven slots provide space for up to twenty-two coverglass preparations, if placed back to back. Stain was contained in polyethylene cups originally manufactured for freezing individual ice cubes. This results in a particularly compact arrangement, each receptacle measuring approximately 2½ cm. by 4 cm.

Fisher and Haskell[6] modified Autotechnicon* and Fisher-Brubach**

* The Technicon Co., Chauncey, New York.
** Fisher Scientific Co., 633 Greenwich St., New York 13, New York (and other branches).

carriers to hold slide preparations for manual immersion in Stender cylinders and Coplin jars respectively. The former holds 13 slides, the latter 5. An advantage of this system is that metal need not contact the staining materials, eliminating the possibility of corrosion and contamination. The slides are held by one end only and do not touch the bottom of the containers.

Good results over a period of twenty years are described by Groat[9] in the use of three Coplin jars for staining blood films. The first jar contained pure Romanowsky stain, the second held stain diluted 1:10 with distilled water and the third was kept filled with clear or nearly clear distilled water for the final rinse.

An automatic staining machine, similar in operation to the Autotechnicon, for use with coverslip films has recently been reported.[5] In staining thousands of preparations during a period of six years, results have been of uniformly high quality. All liquids are contained in jars of 80 ml. capacity. First, absolute methyl alcohol is allowed to act during a 4 min. period for fixation. The machine then lifts the carrier and transfers it to a container of Jenner's stain, diluted with an equal volume of pH 6.8 phosphate buffer, covering the films for 6 min. This provides optimum staining of red corpuscles and leukocyte cytoplasm. The films are next immersed in Giemsa's stain diluted 1:10 in pH 6.8 phosphate buffer for 6 min., emphasizing the staining of chromatin and the granules in neutrophils and platelets. The following step is immersion for 4 min. in phosphate buffer, which is aerated to maintain a constant washing action to remove any scum. Normally, air-drying is used, but a quick rinse in distilled water, followed by blotting dry may improve the final differentiation of structural details. It is indicated that the machine supplanted a hand method using four Coplin jars.

The immersion method, in various forms, has been used in the study of malaria[19] and of trypanosomiasis,[11] mentioned by a veterinarian,[18] included in additional articles[12, 13, 14, 17, 20] as well as in texts.[3 I & II, 4 II, 15] Total elapsed time for staining has ranged from about 10 sec. to overnight—the latter being employed with a 1:30 dilution of Strumia universal blood stain.

Thick films of the type used in examining for parasites present a problem[2] since flaking may occur during the staining process, with subsequent deposition of these detached particles on other slides. However, with the use of ordinary thin films no record of difficulty has been found.

Triton X-100*, a non-ionic liquid detergent, added in 0.01 per cent (v/v) final concentration to Giemsa's-buffer solution results in cleaner bulk preparations and more distinct morphological details of a number of blood parasites.[13] Also, by lowering the surface tension, any detached parasites tend to sink to the bottom of the container rather than adhere to another slide. Higher concentrations caused the red corpuscles to appear pale, hazy and partially de-hemoglobinized.

A blast of warm air from a motor blower with heater has been utilized in drying stained and rinsed slides.[10] The use of heat must be approached cautiously, however; some authors advise against it completely. Bibulous paper was used in the present trial, but drying as well as staining in bulk is an apparent improvement.

* Rohm & Haas Company, Washington Square, Philadelphia 5, Pennsylvania.

The immersion method affords these advantages:

Uniformity of staining. More significance can be attached to minor degrees of shading, since the films in a given tray are under identical conditions (with the possible exception of substances which might be eluted from the films themselves). Various batches of films are consistently comparable in their staining characteristics.

More detail apparent. Although no specific study of this point was made, the impression was gained of cleaner, brighter preparations, with fine distinctions being more clear-cut. Fine basiphil stippling, for example, is considerably more discernible. (The term *basiphil* is used synonymously with *basophilic*, the latter being a less correct Latin derivative.)

Lack of precipitate. While under certain conditions a small amount of precipitate may be deposited in local areas, interference from this source is minimal.

Convenience in placing dishes. Since no sink, pan or water supply is required, the staining equipment may be placed beside the microscope or in any space where staining may be integrated efficiently with other activities. The inflammable nature of alcohol is to be considered, however, particularly as to what would occur in the event of breakage or spillage. In a large institution, it appears feasible to place small containers on the hematology tray so that in instances where speed is essential, staining could be performed *en route* from the patient to the laboratory.

Reduction of time and manipulation. The expedience of handling slides in batches is apparent. Furthermore, it is not necessary to clean their under-surfaces. The tray used in the present trial holds up to twenty slides back to back; up to nine extra may be included if they are placed slightly diagonally.

Reduction of loss and breakage.

Less stain used.

Non-critical timing.

MATERIALS AND METHOD

The following is a slight modification of the method described by Pontius.[16] Since staining trays and dishes of the type shown in Fig. 1 were used, all quantities given are suitable for this type of equipment. The films are submerged consecutively in four materials: absolute methyl alcohol, Wright's stain, phosphate buffer and distilled water.

REAGENTS

1. **Absolute Methyl Alcohol.** Many directions for the preparation of Romanowsky stains specify a neutral acetone-free grade and this type was used in the present trial.

2. **Wright's Stain.** All glassware must be completely dry for preparation and storage. Thoroughly grind 3.0 gm. of powdered Wright's stain in 30 ml. of chemically pure glycerin. Add, in small portions, 1800 ml. of neutral, acetone-free methyl alcohol, grinding with each addition until the stain is dissolved. Aging for six to eight weeks at room temperature allows a margin of time. Storing at 37° C tends to speed the aging process. Filtering before use is not necessary.

3. **Buffer.** Dissolve 4.70 gm. of Na_2HPO_4 (anhydrous) and 4.58 gm. of KH_2PO_4 in 1 l. of distilled water. This results in a M/15 solution of

pH 6.8. There are a number of compounds that may be named "sodium phosphate." Since they are not completely interchangeable, the actual formula must be noted. Some substitutions may be made among the dibasic group if the weight given for Na_2HPO_4 (anhydrous) is first multiplied by a factor to compensate for the water of crystallization. Factors for three kinds of sodium phosphate are:

$Na_2HPO_4 \cdot 2\ H_2O$	1.25
$Na_2HPO_4 \cdot 7\ H_2O$	1.89
$Na_2HPO_4 \cdot 12\ H_2O$	2.52

For example, to use $Na_2HPO_4 \cdot 2H_2O$ in the preparation of the first-mentioned buffer, $4.70 \times 1.25 = 5.88$ gm. $Na_2HPO_4 \cdot 2\ H_2O$ required. If $Na_2HPO_4 \cdot 12\ H_2O$ is exposed in a layer to ordinary atmosphere for two weeks, it loses much of its water of crystallization and becomes $Na_2HPO_4 \cdot 2\ H_2O$. Should there be any doubt as to the amount of H_2O a supply contains, such as might occur in the use of sodium phosphate which had been stored for a long period, the final pH of the buffer should be determined.

For a stock supply, buffer may be prepared as a ten-times concentrated solution, warming, if necessary, to dissolve. The concentrate is conveniently diluted by adding 25 ml. to the dish and diluting in the dish to a permanent mark made at the 250 ml. level. Storage in a polyethylene bottle at refrigerator temperature is preferable. Buffers stored in glass bottles at room temperature have formed a sediment after a few weeks.

Another means of having buffer readily available is to prepare a number of packets each containing the 1.17 gm. of Na_2HPO_4 (anhydrous) and 1.14 gm. of KH_2PO_4 necessary for 250 ml. of pH 6.8 solution. Grinding, before weighing, speeds the dissolving process.

Also, buffer tablets are available* ** *** in 100 ml. and 1 l. sizes for a wide range of pH values.

Although packets or tablets were not used in the present trial, in retrospect they appear to have distinct advantages over storage in liquid form.

Should the use of the pH 6.8 buffer result in staining which, due to particular laboratory conditions, does not fulfill the necessary criteria, hematology texts[4,21] and physiological chemistry tests supply directions for preparing phosphate buffers (Sørenson's or M/15 for additional preliminary trials. pH values of 6.4, 6.5, 6.6, and 6.7 are often compared; rarely pH 7.2 or 7.4 has been employed. It appears advantageous, if *fine* adjustments need be made, to allow two to three weeks use of the staining procedure before any changes are instituted. In performing trials, the distilled water should be renewed each time the buffer is changed, since small amounts of buffer are carried over into the water.

Preparation of films. For optimum results it is necessary to employ completely clean slides, as is customary in technics of this nature. Precleaned slides from two sources have been used and found suitable.

*Coleman Instruments, Inc., Maywood, Ill. (Buffer tablets for 100 ml. of pH 6.4, 6.6, 6.8, 7.0, 7.2 or 7.4, as well as many other values.)

**Edward Gurr Ltd., 42 Upper Richmond Road West, London, S. W. 14, England. (Buffer tablets for 100 ml. of pH 6.5, 6.6, 6.7, 7.0, 7.2, 7.3, or 7.4, as well as many other values.)

***George T. Gurr Ltd., 136/138 New Kings Road, London, S. W. 6, England. (Buffer tablets for 100 ml. or for 1 l. of pH 6.4, 6.8, 7.0 or 7.4, as well as many other values.

Nearly all of the films were prepared by the usual method which results in a thick-thin layer of blood ending in a "fish-tail" (Fig. 3), an effort being made to keep the film from the edges of the slide. The film should be as even as possible, thin, smooth and free of open or irregular areas.

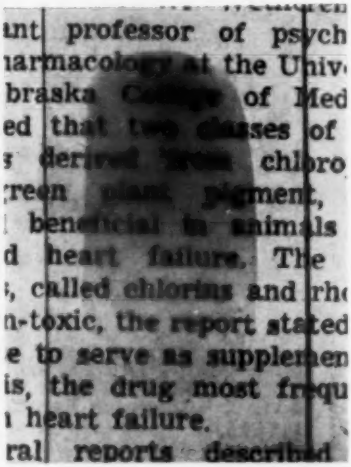

Fig. 3. Thickness and form of a representative film. Only the thinner portions were examined.

A few films were made by a modification of the coverslip technic, in which two ordinary slides were used instead of coverslips and pulled apart along their long dimensions. Two small drops of blood were placed along the long axis of a slide, one drop about 1¾ in. from the short, lefthand side of the slide, and the second drop of blood about ½ in. to the right of the first. The second slide was placed on top of these, with about 1 in. overhanging to the right, and drawn to the right before the drops of blood had spread completely. If the drops of blood are too large, there is considerable overlapping of the red corpuscles, with only a few available for study. Ordinarily, however, all of the blood forms a thin, uniform film, greatly increasing the area usable for examination.

With either method of preparing films, areas which are much too thick appear blue grossly after staining, instead of the correct pink.

Taking precautions against stray fragments of glass, the coverglass-type of film may be subdivided by means of a glass cutter into smaller sections approximately 1 cm. by 2.5 cm. Although not always symmetrical, these semi-micro slides permit performing some five different staining or histochemical procedures from one full-sized original film. Cleanliness of cutter and working area should be observed. After staining or other treatment by means of forceps and small beakers, these semi-micro slides may be examined microscopically by affixing them to full-sized slides with a drop of immersion oil.

Staining. The dishes must be so placed and the trays so handled that there is no possibility of buffer and/or water contaminating the stain, which would ultimately render it useless. The methyl alcohol

must also be protected in this respect. Placing the dishes in a tray approximately 12 in. by 18 in. with paper towels covering the bottom affords a clean area for setting unused staining trays and gives some protection in case of spillage.

Condensates of water may form on the lids of the buffer and distilled water dishes, making it important that the lids be so replaced that drops do not fall into the alcohol or stain. To prevent excessive transfer of liquids from dish to dish, the tray should be drained somewhat before being placed in the next material. However, the draining of the stain should not be so prolonged as to approach the drying point. The tray and slides must be grossly dry before starting the procedure. If the handle of the tray is disengaged it may be difficult to re-insert with the openings covered by the colored liquids.

The time of immersion in the various materials is as follows:

Absolute methyl alcohol	10-20 sec.
Wright's stain	1½- 2 min.
Buffer (diluted, if stocked as concentrate)	2 - 3 min.
Distilled water	
Raise and lower the films until they or their edges become pink. Usually	3 - 5 sec.

While timing does not seem to be critical, it may, of course, be adjusted for optimum results. Slides left intentionally in the stain for 1 hour showed no ill-effects; on being left overnight, though, there was some degradation. The effect of the other materials was not tested in this manner. Ordinarily, however, a staining schedule is followed. Experience with marrow specimens has been somewhat limited; timing appears to be the same as for blood films.

Replenishment of materials. Since the lids of the dishes did not form a tight seal, a heavy layer of silicone stopcock grease was maintained on the lip of the dishes in an effort to reduce evaporation.

The methyl alcohol is kept up to volume by small additions from time to time; it has been changed entirely about once a month as a precaution against excess water entering the stain. The alcohol, as well as the stain, will absorb a certain amount of moisture from the air.

The stain has been used for somewhat longer periods, small amounts being added, when necessary, to keep the surface above the slides. Under ordinary circumstances, renewal every few months should be adequate to prevent excessive water accumulating from the moisture in the air. Methyl alcohol carried over from the first dish compensates to a certain extent for that lost through evaporation.

It has been found advantageous to use the buffer as long as possible, again adding small amounts from time to time. Should scum become bothersome or should precipitated stain on the slide approach the objectionable point, the buffer should be completely changed and the dish wiped free of sediment. Another indication that the buffer may need replacement is the beginning loss of the films' brilliant quality—the eosinophils, for example, appearing slightly dull.

The distilled water has been changed every two to three days, preferably not on the same day that the buffer is changed. The system has been to change the water if the staining began to wane. If this did not correct the color, then the buffer was also changed. After a few weeks

use it becomes possible to devise a schedule which will anticipate these changes and result in minimum deviations in staining.

DISCUSSION

Unquestionably, hematology assigns an important role to the detailed study of a well-prepared blood film. This examination may be considered a form of exfoliative cytology with the possibility of encountering any of a number of abnormal forms. The distinctions among these and from the normal may be slight and subtle, so that any procedure which increases the differentiation is, *ipso facto,* to be sought. On the other hand, even so distinctive a cell as an eosinophil may require close scrutiny for identification if conditions are not adequate. Several months trial of the immersion method under the usual, day-to-day conditions of the laboratory has demonstrated many advantages without revealing any undesirable aspects.

The differential count being the culmination of a series of steps, beginning with the blank slide, the chain is no stronger than its weakest link. Any one of a number of seemingly small details may be a source of difficulty. To compensate for this delicate situation is the fact that evaluating the results of the complete procedure is a matter of simple microscopic examination, with adjustments readily made.

The thickness of the film has some effect on the staining results, thin preparations being much superior. Ideally, there should be a considerable area in which the corpuscles nearly touch each other, without overlapping, and there should be minimal aggregation of structures at the edges of the film.

A number of cleansing agents have been recommended for used slides or those not pre-cleaned. Initially, dichromate cleaning solution, soap (do not allow the soapy water to cool) or detergent may be used to remove the bulk of unwanted material, with rinsing followed by acetone, xylol, dilute acetic acid or glacial acetic acid. This is followed by a thorough rinsing in tap, then distilled water, after which they may be stored in ninety-five per cent alcohol until polished just prior to use. Alternatively, after polishing, slides may be tightly wrapped in paper and stored until used. Flaming is a means of removing last traces of grease.

Each laboratory should determine which method gives the best results under its particular conditions. The use of detergent for cleaning slides, for example, although recommended by some, will, in certain circumstances, cause films to spread poorly. Once a satisfactory overall system has been determined, it should rarely, if ever, require adjustment as long as there is adherence to all aspects.

The criteria of satisfactory staining by immersion are the same as those of other Romanowsky technics. Grossly, as previously indicated, the film should be pink, except in the unavoidably thicker areas, which appear blue. Microscopically, in the normal, ideal film, the red corpuscles appear a salmon color. Nuclear material (except that of monocytes) is dyed purple. The darkest portion (granulomere) of platelets becomes a violet or purple, while the hyalomere is pale blue with azure granules. Neutrophil cytoplasm has a pinkish tint, with purple granules; eosinophil granules are pink. The cytoplasm of lymphocytes and monocytes appears blue. The overall bluish tinge of young red corpuscles, as a

sensitive index of proper staining, should be demonstrated before the system is considered properly adjusted. Parenthetically, it should be pointed out that there is not universal agreement on the terminology of this bluish tinge. It is known, variously, as *diffuse basophilia* (or *basiphilia*), *polychromasia,* or *polychromatophilia;* the last two terms are often used by British authors. In the United States, some authors restrict *polychromatophilia* and *polychromasia* to the description of red corpuscles having within the single corpuscle areas both of bluish tinge and of pink.

If the neutrophil nuclei and the platelets stain only palely, the pH may be too acidic, the stain not sufficiently aged, or the time in the stain and/or buffer may be too short.

Overstaining should not be a source of difficulty with the present method. If the structures appear overly blue, the buffer may be too alkaline; conversely, a tendency to excessive redness is found if the buffer is too acidic.

There has been no indication of structures' being transferred from one slide to another. The possibility of such an occurrence appears extremely remote. To cause an error, it would be necessary for an object such as a cell to become detached from a slide, then for another object of very nearly the same size and shape to be deposited in precisely the same area. With uneven or excessively thick films, detection might not be quite so simple, although any added object would probably focus, with oil-immersion, in a plane above the film.

On no slide was there any evidence of flaking or peeling. With ordinary handling, a blood film adheres very well to the glass. Often, slides are stacked or handled without particular care even before completely dry and have been, generally, for years, without any signs of structures being interchanged. If there were any tendency of this kind, it should be well documented by the present time.

In the extensive use of the automatic staining machine described by Davidson and co-workers, there is no mention of any interchange despite the fact that on some occasions marrow smears were processed. Due to the fat content, marrow smears should, possibly, be less adherent. The exact means by which the coverslips are held during the process is not described, but there is no indication that any particular precautions were taken.

In some immersion methods, films are stained in aqueous solutions without previous fixation, with no difficulty reported.

Transfer of structures from one slide to another appears to be limited to the special case of thick smears. Occasionally the usual thick-thin film will be so dense in the beginning central area that there might be the possibility of flaking. This, of course, must be avoided.

Nevertheless, as long as there is more than one film in a liquid, there is the theoretical possibility—although extremely remote—of the exchange of cells or other material. One factor which increases this possibility slightly is the rubbing or scraping of any film which may be on the ends of the slide. Also, overconfidence might lead to insufficient care in handling.

The slide holder shown in Fig. 2, it is to be noted, permits manipulating the films without their coming in contact with any surface which

might tend to scrape them, resulting in loose particles in the fluid. Ordinarily, at least one end of a slide is free of film. By placing this end in the holder, the films need contact nothing other than the liquids. The plastic bar resting on the lip of the jar is not part of the original equipment, but was added to elevate the slides above the bottom of the Stender jar. Several holders might be placed on a longer bar to increase the capacity of the system. Alternatively, the modified Autotechnicon carrier of Fisher and Haskell could be employed.

On theoretical grounds, the ideal type of equipment is that which permits nothing but the staining materials to come into contact with the film.

In initiating the system, using new dishes and previously unused materials, it has been noted that the staining, while adequate from the beginning, has benefited somewhat by a two to three week period of use. The effect of aging on certain aspects of staining is well known; possibly this increase in acuity is the result of an equilibrium's being attained among the staining agents and between the agents and the glass.

In view of the wide variety of stains which have been used in immersion methods, it would appear that this technic is applicable to any of the Romanowsky stains, as well as other types. Giemsa's or MacNeal's[1] stains, being of more or less known, invariable composition, might provide an even higher degree of uniformity in results than Wright's and the other Romanowsky stains which may vary from lot to lot in production. MacNeal's stain is composed of:

Eosin, yellowish, water soluble	1 gm.
Methylene blue, medicinally pure	1 gm.
Methylene violet (Bernthsen)	0.2 gm.
Methylene azure (Azure A)	0.6 gm.
Methyl alcohol, absolute, acetone-free	1000 ml.

In listing the ingredients of MacNeal's stain it is customary to specify the methylene blue as "medicinally pure." Apparently, this is the ordinary methylene blue used in many staining procedures. The above substances are placed in a 50° C water bath, avoiding open flame, and thoroughly shaken. The mixture is then left at 37° C for a day or two with occasional shaking to complete the solution. For aging, three to four days incubation at 37° C has been recommended. This stain is also available in powder or tablet form, sometimes being known as Tetrachrome Stain (MacNeal). MacNeal's stain is used in a manner similar to Wright's.

SUMMARY

1. A previously described immersion technic for Wright's staining blood films has been modified slightly and employed in the daily laboratory routine. Using slide trays and staining dishes, thin films of blood are submerged consecutively in four materials: absolute methyl alcohol, Wright's stain containing 1.6 per cent glycerin, M/15 phosphate buffer of pH 6.8, and distilled water.

2. Results have been uniform and of higher quality than attainable by the use of a staining rack. The use of Giemsa's or MacNeal's stains might provide even more uniformity.

3. There has been no evidence that structures are transferred from one slide to another, even though no particular precautions were taken. Certain types of equipment have theoretical advantages in this respect.

4. Since films can be processed in batches of twenty or more, the method requires considerably less time and manipulation. The equipment may be located near the microscope or in any convenient area.

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THE OCCURRENCE OF THE LE CELL PHENOMENON*

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INTRODUCTION

Since the recognition of the LE cell in concentrated films of heparinized bone marrow by Hargraves et al in 1948,[14] a variety of techniques for the demonstration of the LE cell has been developed. The significance of the LE cell has been under study for ten years. During this period, the reliability and specificity of the LE cell phenomenon and the sensitivities of the various methods have been examined, while at the same time there has been an apparent increase in the incidence of, or diagnosis of, systemic lupus erythematosus. In addition, the LE cell has been reported to occur in certain other clinical conditions, and thus the specificity has been questioned.

It is the purpose of this paper to attempt to summarize some of the findings, reported in the literature, relating to methods for demonstration and interpretations of the LE cell, together with results observed in our laboratory.

REVIEW OF THE LITERATURE

Techniques. Shortly after Hargraves' definition and description of the LE cell,[14] his observations on heparinized bone marrow in patients with systemic lupus erythematosus were confirmed by Haserick and Sundberg[17] and the significance of the LE cell with regard to this diagnosis was emphasized. Subsequently, in the attempt to understand the phenomenon as well as to simplify techniques for its demonstration, methods have been developed from the original observations. Sundberg and Lick[55] and Hargraves[15] in 1949 reported LE cells in concentrated leukocyte (buffy coat) films of venous blood from patients with systemic LE. Plasma from patients with systemic LE, when mixed with non-lupus bone marrow, was found to induce the phenomenon by Haserick[18] and Hargraves[15] in 1949; the LE cells thus formed were identical with those seen in concentrated bone marrow or blood buffy coat films. LE cells were demonstrated in clotted blood from patients with systemic LE by Barnes et al[1] and Gonyea et al[13] in 1950. In 1955, the phenomenon was demonstrated when plasma or serum (Schultz[50]) or blood (Snapper and Nathan[53a]) from a patient with systemic LE were used to flood a ring of dried leukocytes from a non-lupus subject.

The methods in common use today for demonstration of the LE cell phenomenon are based on these fundamental techniques. LE cells have not been noted on direct smears of blood or bone marrow, with the exception of one reported case in which they were seen three days antemortem from direct films of capillary blood.[5] Sickley et al have succeeded in demonstrating LE cells from finger-tip blood after constriction of the finger,[51] but they have shown that the time of constriction is a determining factor in this procedure.[52]

The use of plasma or serum from patients with systemic LE together with non-lupus bone marrow as an **in vitro** test for demonstration of the LE cell led to the implication of a specific factor in the plasma

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gamma globulin fraction of patients with systemic LE.[3, 19-21] This factor was shown to be immunologically distinct from normal gamma globulin, and could be removed from solution by immunological precipitation.[20] The reaction between the LE factor and substrate cells was found not to be species specific,[3, 21] Carrera et al[4] compared the relative sensitivities of a variety of animal species in this method, and concluded that human bone marrow was relatively low in susceptibility to the activity of LE serum, while chicken and horse marrow were the most susceptible. The use of blood rather than bone marrow as substrate material has also been introduced.[21, 33, 41] Rohn et al[48] have demonstrated that human leukocytes from certain diseases, when used as substrate material, exhibit a striking amount of erythrophagocytosis which overshadows the formation of LE cells; in a second group, erythrophagocytosis and the LE cell phenomenon were seen with approximately equal frequency, while in still a third group as well as in normal material, the LE cell phenomenon predominated.

The demonstration of LE cells from clotted blood ruled out anticoagulants as necessary participants in the formation of LE cells. Indeed, anticoagulants appear to exert an inhibitory effect.[8, 24, 33, 35, 61] Zimmer and Hargraves[61] demonstrated that, when clotted blood was used, the optimal time between withdrawal of blood and preparation of slides was two hours, and the two hour clot test has become a widely used method. The use of a wire sieve[37] to macerate the clot in this method is convenient, and it is possible that the trauma thus introduced is of some importance in the sensitivity of the test.[8] Defibrination of blood has also been used in the demonstration of LE cells.[9, 38]

The role of the coagulation process itself in the formation of the LE cell is not established. Because of the apparent enhancement of the phenomenon after blood had clotted,[61] coagulation was postulated to be a necessary factor to the optimal formation of LE cells, and anticoagulants, at least in excess, have been demonstrated to be inhibitory.[8] Lee et al[35] reported a thromboplastin factor present in platelets to be associated with the formation of LE cells. The use of siliconized glassware has been shown to be inhibitory.[35, 62]

An important modification of the peripheral blood buffy coat method utilizes heparinized blood which is rotated with glass beads (Zinkham and Conley[62]), and thus introduces trauma to the participating elements. Dubois and Freeman[8] emphasized that trauma to the cells, as achieved by rotation with blass beads or by forcing clotted blood through a wire sieve, rather than the process of coagulation, was responsible for increased sensitivity in the demonstration of LE cells. In the sieved two hour clot method, blood is allowed to stand for two hours at room temperature. The clot is then forced through a wire sieve* with a brass pestle, the fluid material is collected in a Wintrobe hematocrit tube, centrifuged for 10 minutes at 2000-3000 RPM, and the concentrated leukocyte layer, together with an equal amount of serum, is mixed and spread on a glass slide. In the method using rotation with glass beads, heparinized blood is allowed to stand at room temperature for 30-90

* Scientific Products Division, American Hospital Supply Corp., Evanston, Ill.

minutes, then rotated with glass beads, 4 mm diameter (approximately 10-20 glass beads per 10 ml blood) at 40-50 RPM for 30 minutes, and the leukocytes concentrated as in the sieved clot method. These two methods, one using anticoagulant and the other involving coagulation, compare well in sensitivity.[8, 62]

Hargraves, in his original observations on the LE cell,[14] reported that the inclusion body gave a positive Feulgen reaction. Lee et al[33] confirmed by cytochemical analysis that the inclusion body of the LE cell is nuclear in origin. He showed that desoxyribose nucleic acid of the LE cell inclusion body had undergone at least partial depolymerization, and he emphasized the similarities of LE cells with the hematoxylin-staining bodies described by Klemperer et al[31] from postmortem material in cases of systemic LE. The derivation of LE cells, from intact neutrophils through various stages of autolysis to the final LE cells, has been observed by Rebuck and Berman[45] and Rohn and Bond.[47]

It is apparent from these miscellaneous observations that certain factors and conditions are necessary for the formation of the LE cells:[3, 8, 15, 16, 19, 47, 54, 61]

1) **LE cell factor,** which resides in the gamma globulin fraction of plasma from patients with systemic LE;
2) **Cell nuclei,** a source of nucleoprotein and the origin of the inclusion body; not necessarily from the patient;
3) **Phagocytic cells,** usually neutrophilic leukocytes; not necessarily from the patient;
4) **Time** for the production of the phenomenon.

Sensitivity of methods. Comparisons of methods have indicated that clotted blood provides a more sensitive method than anticoagulated blood or bone marrow from the patient, or plasma or serum with non-lupus bone marrow.[8, 24, 34, 38, 61] Haserick[24] reported that, although patients with classical systemic LE showed LE cells by any method, 33 of 79 patients with "atypical" systemic LE had positive results with one or more of the three methods used: clotted blood was positive in 30 cases, concentrated bone marrow was positive in 24 cases, and plasma plus dog bone marrow was positive in 19 cases. He suggested the likelihood that excessive heparin was inhibitory in the latter two methods.

Zinkham and Conley showed that their method, in which heparinized blood is rotated with glass beads, compared favorably with the two hour clot method.[62] Since these authors demonstrated that the production of LE cells was markedly greater when blood was rotated with glass beads than when either rotation or glass beads were omitted, or when siliconized glassware was used, they suggested that the enhanced effect of rotation with glass beads was due to trauma to the cells, and that viable leukocytes are probably not subject to the influence of the LE factor.

Dubois and Freeman[8] compared some of the available methods in two groups of experiments. In the first series of 68 batteries on 44 established cases of systemic LE, the two hour clot method was positive in 60.3%, the Snapper ring method, in 42.7% and the heparinized buffy coat method, in 36.8%. In the same series, although the clot method showed the only positive results 10 times, the Snapper ring method showed the only positive result 4 times and the heparinized buffy coat

method, 2 times. A definite inhibition with excessive heparin was demonstrated. In the second series of 63 batteries on 47 established cases of systemic LE, the method of Zingham and Conley was included. This method, using rotation with glass beads, was positive in 81%, the clot method, in 74%, the Snapper ring test, in 62%, and heparinized buffy coat, in 35.8%. Each of these showed the only positive results 5, 4, 2 and 0 times respectively. They noted no case in which there was a negative result with peripheral blood and positive results with bone marrow. They also reported an increase in the number of LE cells produced in a given specimen when the wire sieve was used in the clot method over the relatively less severe maceration of the clot with wooden applicator sticks.

Reliability of LE cell phenomenon. Reports have consistently indicated that LE cells can be found with one or more methods in the majority of cases of systemic LE.[7, 10, 24, 33, 50, 54, 57, 59] The figures, when given, usually indicate that from 65% to 96% of such patients have positive tests at some time during the course of the disease. The apparent increase in the incidence of systemic LE may be due in part to the use of these methods as a diagnostic tool.[7, 16, 22]

The effect of therapy and severity of the disease on the demonstration of LE cells has been variable. Some[38, 57] have expressed the opinion that the presence and number of LE cells is an indication of the severity of the disease. A marked decrease or disappearance of LE cells after treatment with ACTH has been reported.[21, 47] Dubois and Freeman[8] reported that approximately 50% of patients had complete or almost complete disappearance of LE cells after two or more months of therapy.

Specificity of the LE cell phenomenon. Early reports were optimistic with regard to the specificity of LE cells. Both large and small series of observations indicated that, while the majority of cases of systemic LE showed LE cells, chronic discoid LE, periarteritis nodosa, dermatitis, rheumatic fever, rheumatoid arthritis, erysipelas, multiple myeloma, scleroderma, dermatomyositis, thrombocytopenic purpura, and various drug hypersensitivities were reported to have negative results.[7, 33, 54, 57, 61] On the other hand, occasional undiagnosed patients with positive LE cell tests were reported as compatible with systemic LE.[21, 61]

However, LE cells have been reported occasionally in a variety of diseases,[3, 28, 36, 42, 60] and repeatedly in a limited number of diseases. The numbers of such cases are small relative to the incidence of positive LE cell tests in systemic LE, but their importance, if they are real, cannot be dismissed. Among the more interesting and regularly recurring reports are those in penicillin sensitivity,[2, 43, 58] postnecrotic cirrhosis,[25, 29] hydralazine sensitivity,[23, 44, 46, 53] and rheumatoid arthritis.[10, 11, 23, 27, 30, 32, 39, 42, 49, 60]

Hydralazine (Apreseline®), an antihypertensive drug, was reported to have caused collagen disease of varying severities in 17 of 211 patients to whom the drug was administered;[44] the manifestations varied from a mild syndrome with arthralgia and laboratory findings of hepatic abnormality, to a more severe form resembling acute rheumatoid arthritis, to a fully developed form resembling systemic LE. One of these patients exhibited a positive LE cell test. The clinical and laboratory findings of these patients have in general been reported to be reversible with withdrawal of the drug.[44, 53] Comens[6] has been able to reproduce

clinical and pathological changes, including the LE cell phenomenon, of systemic LE in experimental dogs administered hydralazine.

From the standpoint of incidence, rheumatoid arthritis is the most important of this group. From 3%[27] to 25%[39] of patients with rheumatoid arthritis are reported to have positive tests for LE cells. Interpretations vary; Kurnick[32] suggested that true LE cells occur only in systemic LE, and those cases of rheumatoid arthritis in which positive LE cell tests occur may represent systemic LE of a milder and more chronic form (while cases of apparent hypersensitivity to drugs with positive LE cell tests may represent systemic LE exacerbated by drugs). However, he stresses the difference between true LE cells and and false or pseudo-LE cells which are chemically or developmentally different. Thus the presence of true LE cells would indicate the presence of systemic LE.[49, 60] Others have suggested the LE cell not to be limited to systemic LE, but rather to be specific for a limited group of diseases, and that cases of rheumatoid arthritis with positive LE cell tests differ from others with negative LE cell tests.[30] Thus the LE cell is not necessarily specific for, but most characteristic of, systemic LE.[42] Friedman[11] concluded that the LE cell may occur in patients with rheumatoid arthritis as a nonspecific reaction of unknown etiology, and stressed that systemic LE should not be diagnosed unless confirmed by other evidence. On the other hand, since the great majority of positive LE cell tests occur in systemic LE, it might be emphasized that evidence for a diagnosis of systemic LE must be diligently sought when a positive LE cell test occurs.[60]

In association with rheumatoid arthritis, positive LE cell tests have been noted after withdrawal of cortisone.[10, 26, 27]

It should be obvious that careful interpretation of LE cell preparations is of utmost importance.[8, 32, 49, 60] The necessity for discretion was indicated as early as 1948 by Hargraves,[14] when he contrasted the LE cell (whose inclusion body appears completely homogeneous and structureless and without evidence for surrounding membrane) with the "tart" cell (whose inclusion body is identifiable as a nucleus). Forms which appear morphologically to be intermediate between these two opposing types are inevitable. Kurnick[32] proposed two types of pseudo-LE cells which can be differentiated from true LE cells: that which is morphologically identical but chemically different (e.g. amyloid), and that which is morphologically and chemically indistinguishable (nuclear in origin), but which has a different pattern of development. The latter evolve from leukophagocytosis of an entire cell or an intact nucleus, which is then digested, while the true LE cell undergoes depolymerization and digestion before phagocytosis. Meyler[40] reported phagocytosis of lymphocytes, which resulted in cells resembling LE cells but with degeneration after phagocytosis rather than before, in severe drug reactions.

MATERIALS AND METHODS

The results of examinations for the LE cell done in the Special Hematology Laboratory of the University of Minnesota Hospitals during the period between 1950 and 1957 were surveyed. All cases in which at least one positive or questionable result was reported, and all cases with a clinical diagnosis of systemic LE, subacute LE and chronic discoid LE

on whom LE tests were done, were included in the survey. No attempt was made to include all of the cases in which only negative results were obtained. A total of 437 tests were included, of which 315 were two hour clot tests, 41 were bone marrow examinations on the patient, 34 were venous blood buffy coat examinations on the patient, and 47 were patients' plasma or serum plus non-lupus bone marrow.

The two hour clot test was done as described by Zimmer and Hargraves;[61] the wire sieve as described by Magath and Winkle[37] was introduced in 1952. Examinations of patients' bone marrow were done from concentrated myeloid-erythroid films of heparinized bone marrow.[58] Examinations of blood buffy coat were done from oxalated venous blood. The plasma or serum plus bone marrow method was done by mixing 0.5 ml patient's plasma or serum with 1.0 ml non-lupus human bone marrow, incubating at room temperature for 30 minutes, centrifuging and preparing concentrated films from the myeloid-erythroid layer.

The material was reported as positive when typical LE cells were seen. A report of questionable, doubtful or suspicious was made when cells resembling LE cells were seen but when typical LE cells could not be found, or when only one typical LE cell could be found.

RESULTS

In some of the cases included in this study, only a single examination was available, while in others repeated examinations were done. All of the tests done on a given patient were included in the survey. No attempt was made to select the results with regard to therapy, and steroids had been administered to many of the patients prior to the examinations. After the introduction of the two hour clot test in 1951, this method was routinely used; other methods were used alternatively when results were doubtful or unsatisfactory with the clot test, or when LE cells were seen in such preparations incidentally.

The clinical diagnoses were obtained from the patients' records, and no attempt was made to reevaluate these.

Results of the LE cell tests are summarized in Table 1. Of the 41 patients with a clinical diagnosis of systemic LE, 157 positive results (101 from clot tests) were obtained on 38 patients, or 93% of the patients with this diagnosis. Three patients with systemic LE had no positive tests for LE cells. Seven patients also had a diagnosis of rheumatoid arthritis.

Of the five patients in whom a diagnosis of systemic LE was considered a possibility, on the basis of suggestive clinical or laboratory findings, all had positive LE cell tests except one, in whom the test was negative four times and questionably positive once. On the four patients with LE cells, 13 positive tests were reported, of which 11 were obtained with the clot test.

Of the six patients with a diagnosis of rheumatoid arthritis, in whom systemic LE was considered as a possibility, all had one or more positive LE cell tests. It is of interest that one of these had a postmortem diagnosis of polyarteritis nodosa, with no pathological evidence for systemic LE. Twenty positive tests were obtained, of which 18 were clot tests.

Table 1
RESULTS OF LE CELL TESTS

Clinical Diagnosis	No. Pts.	Female/ Male	Age Range (Yrs.)	Adults/ Children*	No. Positive LE Cell Tests	No. Questionable LE Cell Tests	No. Negative LE Cell Tests	No. Pts. With No. Pos. Tests
Systemic LE	41	36/5	14–65	36/5	157	21	89	3
Systemic LE possible	5	3/2	15–67	4/1	13	2	9	1
Rheumatoid arthritis Systemic LE possible	6	5/1	38–56	6/0	20	2	12	0
Rheumatoid arthritis No evidence for systemic LE	7	5/2	16–66	7/0	6	4	15	3
Miscellaneous No evidence for Systemic LE	8	6/2	15–59	7/1	0	12	26	8
Subacute LE	9	8/1	15–81	8/1	0	2	34	9
Chronic discoid LE	8	5/3	33–75	8/0	0	0	13	8

*Children = to 16 years.

LE cells were seen in four patients with a diagnosis of rheumatoid arthritis, with no clinical evidence for systemic LE. Of six tests done, five were clot tests. Although many patients with a diagnosis of rheumatoid arthritis have had only negative tests for LE cells, not all patients with this disease have had examinations for LE cells. Therefore, no conclusive data can be presented from this material regarding the relative incidence of positive LE cell tests in rheumatoid arthritis. Questionable LE cell tests, but no positive tests, were reported on three patients in this group.

Eight patients with a variety of clinical diagnoses, without evidence for systemic LE, had questionably positive but no positive LE cell tests. Of twelve questionable tests, ten were clot tests. Two of these patients had chronic post-infectious hepatitis and two others had pathologic evidence for polyarteritis nodosa. One patient was thrombocytopenic, possibly due to phenobarbital or Reserpine.[R] One had a postmortem pathological diagnosis of chronic glomerulonephritis; one had chronic pancreatitis; one had a pleural effusion, etiology unknown.

Nine patients with a diagnosis of subacute LE were found, of whom none had positive tests for LE cells, while two had questionable clot tests. Eight patients with a diagnosis of chronic discoid LE had only negative tests for LE cells.

In only one instance was a negative clot test reported when an alternate method (serum plus non-lupus bone marrow), done on the same day, was positive. This was found in a case of established systemic LE. No absolute comparisons among methods can be made from this material, since, of the 437 tests done, 315 were clot tests.

DISCUSSION

The methods of choice for demonstration of LE cells have been shown to be the two hour clot test of Zimmer and Hargraves and the method of Zinkham and Conley using rotation with glass beads. These methods are more sensitive than other available techniques, and they are relatively convenient to both laboratory and patient. Methods using plain anticoagulated blood or bone marrow, or plasma or serum plus non-lupus blood or bone marrow, however, have the advantage that mor-

phology of the LE cells is relatively good, and such cells are often more easily identifiable or less equivocal than similar cells demonstrated by preferred methods. In addition, concentrated films of blood or bone marrow are often examined for reasons other than determining the presence of LE cells, and these abnormal elements may be accidentally discovered. One of the more sensitive methods should, therefore, be used in the routine examination for LE cells, with one or more alternate methods to be used when indicated.

Although the data now available indicates that the LE cell phenomenon may occur in conditions other than systemic lupus erythematosus, the phenomenon nevertheless appears to be limited to a relatively narrow group of diseases. The large majority of cases in which LE cells are demonstrable is systemic LE (and conversely, the large majority of cases of systemic LE demonstrates LE cells), while LE cells occur only in occasional cases of the other categories. The most important of these are rheumatoid arthritis, with a lesser number occurring in drug hypersensitivities such as penicillin or hydralazine reactions.

It does not seem likely that those cases other than systemic LE in which LE cells are seen should be thought of as indiscriminate false positives, but rather the LE cell phenomenon appears to be a more or less specific reaction occurring in a restricted group of clinical conditions. This seems particularly reasonable since, in recent years, the definition of what do and what do not constitute LE cells is more secure with the accumulating experience with the phenomenon and with methods for its demonstration. The value of the test is not compromised. In spite of the occurrence of LE cells in approximately 5% of patients with rheumatoid arthritis, Frerichs[10] has stated that a positive LE cell test is the most reliable laboratory means of distinguishing between systemic LE and rheumatoid arthritis.

A test should be called positive only when typical LE cells are seen.[8, 60] Although leukocyte clumping and extracellular basophilic material are significant enough to indicate an exceedingly careful search, they do not constitute definitive evidence for the LE cell phenomenon. The typical LE cell must be carefully defined and the criteria for identification rigidly observed.

SUMMARY

1. The two hour clot method of Zimmer and Hargraves and the rotation method with glass beads of Zinkham and Conley are the methods of choice for demonstration of the LE cell phenomenon.

2. LE cells may occur in a limited group of diseases other than systemic lupus erythematosus, the most important of which is rheumatoid arthritis. The majority of positive tests, however, occurs in systemic LE, and the majority of cases of systemic LE demonstrates LE cells.

3. Criteria for identification of the LE cell phenomenon must be strictly observed, and typical LE cells must be seen before a test can be called positive.

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CORRECTION

"Simple Rapid Technic for Serum Total Cholesterol," by Mrs. Evelyn Abell, MT (ASCP). January-February 1959, page 66, *Procedure, add number (3) 0.2 ml. SERUM.*

Pipette into 125 ml. Erlenmeyer Flask:

(1) 4.6 ml. CHOLESTEROL REAGENT
(2) 0.9 ml. SULFURIC ACID
(3) 0.2 ml. SERUM

ON THE ISOLATION OF LISTERIA MONOCYTOGENES FROM BIOLOGICAL SPECIMENS*

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INTRODUCTION

In the past ten years there has been a rather striking upsurge of interest in diseases of animals and man due to *Listeria monocytogenes*. This is quite apparent from the literature and in the number of investigators throughout the world now engaged in research on this subject. Yet, in spite of increased knowledge gained during this time concerning the organism itself and various clinical aspects of listeriosis, little progress has been made on certain public health aspects of the disease, e.g., natural habitat(s), sources of infection, vectors, carriers, asymptomatic disease, and avenue(s) of infection for man and other animals. Research on these problems has been hampered considerably by the lack of a reasonably good method of rapid isolation of the organism from the mixed microbial flora of such specimens as stool, soil, vegetable infusions, animal feed, contaminated biopsy and necropsy material, etc. This paper will describe a procedure, which in our opinion has the degree of sensitivity and accuracy needed to attack some if not all of the problems mentioned, even by those investigators unfamiliar with *Listeria monocytogenes*.

MATERIALS AND METHODS

(1) A sterile mortar and pestle, blender or similar apparatus by which solid material may be triturated.

(2) Screw-capped 20 x 150 mm test tubes containing about 9 ml. of difco tryptose broth.

(3) **Modified McBride's Medium**[1]

Difco phenyl ethanol agar	35.5 gm.
Glycine, Anhydride	10.0 gm.
Lithium chloride	0.5 gm.

Dissolved with the aid of heat in 1000 ml. of distilled water and sterilized at 121° C for 20 minutes.

(4) **Modified McBride's Medium with Aci-dione**** (for contaminated material). The medium is prepared as in No. 3, with Acti-dione added in a concentration of 200 micrograms per milliliter. Both of these media are used in the same manner except that specimens suspected of being grossly contaminated are inoculated onto the Acti-dione medium.

The original medium of McBride[1] was modified so that the technique of Gray[2] could be applied in the examination of material suspected of containing listeria. The plates are inoculated with pretreated material, incubated 24 hours at 37° C and examined with a binocular scanning (10 to 15X) microscope employing obliquely transmitted light. (Fig. 1). The listeria colonies, if present, are slightly raised, with a finely textured surface and *blue-green* in color.[2]

* A contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Canada. Received for publication October, 1958.

** Registered trademark of the Upjohn Company.

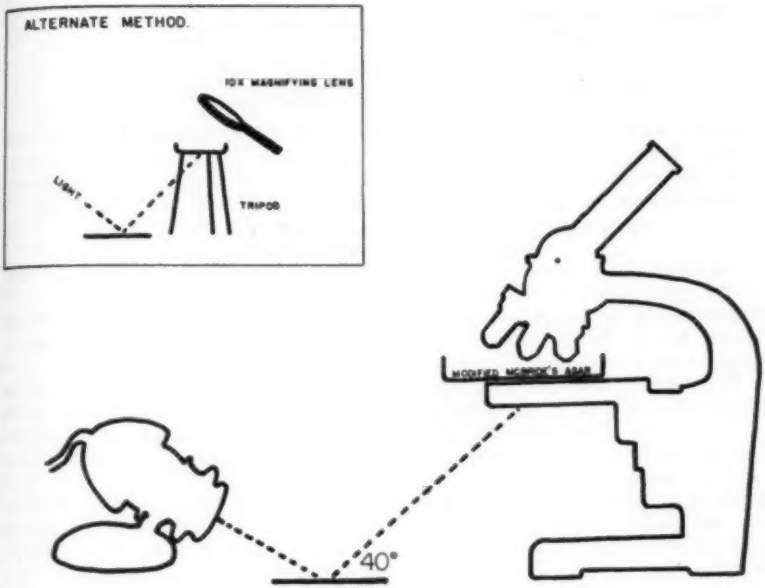

Fig. 1.

(5) **Motility Test Semi-solid Medium**

Difco Bacto tryptose	10.0 gm.
Sodium chloride	5.0 gm.
Agar	5.0 gm.
Dextrose	0.1%

Dissolved with the aid of heat in 1000 ml. of distilled water, distributed in tubes and sterilized at 121° C for 20 minutes.

Suspected colonies are inoculated into the semi-solid by stab inoculation and examined, after 24 hours incubation at room temperature, for type of growth. Listeria show a characteristic type of growth in dextrose semi-solid agar stabs. The organisms, due to their motility, diffuse rapidly throughout the medium. A maximum zone of growth appears about half a centimeter below the surface of the medium.

(6) **Anton's Test for Listeria Monocytogenes.**[3]

A diagnostic charactertstic of listeria is the production of a keratoconjunctivitis in the eye of a rabbit or guinea pig when a freshly isolated strain is inoculated by gently swabbing the inside of the lower eyelid. The animal is examined over a period of ten days. Smooth cultures of listeria produce a purulent conjunctivitis within five days. Keratitis is detected by development of corneal opacity following the conjunctivitis.

(7) **Fermentation Broth**

Beef extract	1 gm.
Difco proteose peptone No. 3	10 gms.
Sodium chloride	5 gms.
Brom cresol purple	0.01%

Dissolve with the aid of heat in 1000 ml. of distilled water and sterilize at 121° C for 20 minutes. Divide the broth into five aliquots and add to each the desired "sugar" in a concentration of 0.5%. The necessary carbohydrates are glucose, salicin, rhamnose, dulcitol and raffinose.

TECHNIQUE OF TREATING SPECIMENS AND METHOD OF ISOLATION

All specimens received in the laboratory should be treated by one of the two methods outlined below.

Method 1. The following specimens should be treated by this method. Blood, spinal fluid, cervical, vaginal and throat swabs, sputum specimens, swabs from boils, urine, pleural fluid, meconium and feces.

The specimen to be examined is inoculated into two tubes of tryptose broth, one of which is incubated at 37° C for 18 to 24 hours, the other at 4° C. The 37° C culture, after incubation, is inoculated onto a modified McBride's medium plate, incubated overnight at 37° C and examined as previously described.

Method 2. Specimens such as brain, liver, spleen, tissues from fetuses etc., which had been removed aseptically by the pathologists are placed in a sterile mortar containing a few grams of sterile sand, 10 to 20 ml. tryptose broth added and the tissue ground to a smooth paste. If a blender is available, the tissue is macerated in 20 ml. of tryptose broth of 1 to 2 minutes. The paste is aseptically inoculated into two tubes of tryptose broth. One of which is incubated at 37° C, the other at 4° C. The 37° C culture is examined as in Method 1.

EXAMINATION OF SPECIMENS INCUBATED AT 4° C

Specimens incubated at 4° C should be examined every week. Listeria grow well at this temperature and reach maximum growth in 7 to 10 days.[4] However, for reasons unknown at present, listeria growth may be inhibited in the presence of tissues, and incubation at 4° C may have to be extended to as much as three months.[2] As a routine procedure the cultures should be examined visually for evidence of growth throughout the broth. When, and if, such growth occurs the cultures are inoculated to McBride's Medium (see Fig. 2).

DESCRIPTION OF LISTERIA MONOCYTOGENES

The description given in this paper is based upon our observations on over 130 strains of *Listeria monocytogenes.*

Listeria monocytogenes, although sometimes difficult to isolate, grows well on ordinary laboratory media. It is a gram positive, motile rod about 2 microns in length and about 0.6 microns in diameter. In culture media it may be slightly curved, occurring singly, in palisade formation, as diplobacilli or in short chains. The organism grows at temperature ranging from 2.5° C to 42° C. It is non acid fast, non-sporeforming and does not show metachromatic granules. The optimum temperature for growth is 37° C, for motility about 20 to 25° C. Most strains show beta hemolysis on blood agar, the colony resembling somewhat that of a beta hemolytic streptococcus.

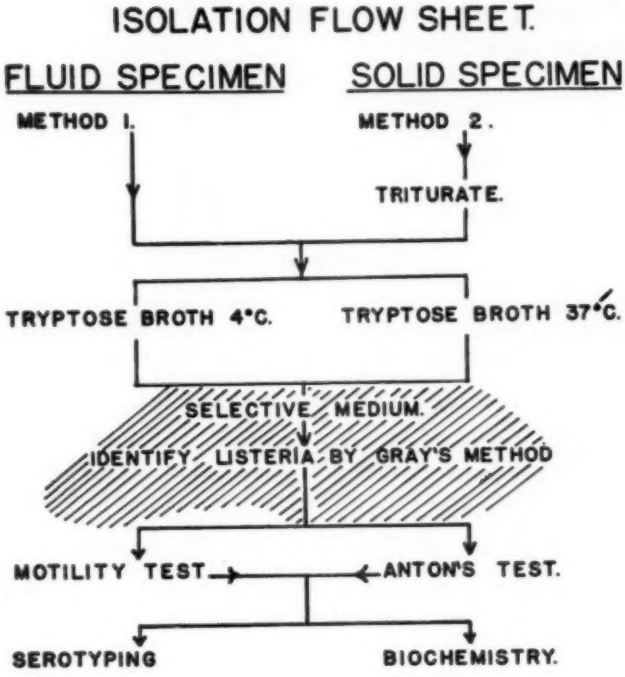

FIG 2.

BIOCHEMICAL REACTIONS

In our experience, the reactions outlined in this section are reliable and not subject to variation due to strain differences.

Glucose	acid	no gas	These carbohydrates are usually fermented in 24 hours, however, they should be observed over a period of one week.
Salicin	acid	no gas	
Rhamnose	acid	no gas	
Raffinose	no reaction		
Dulcitol	no reaction		

Indole not produced
Urea not hydrolysed
Catalase production positive.

Nitrates not reduced
Gelatin not liquefied

ANTIGENIC ANALYSIS OF LISTERIA MONOCYTOGENES[5][6]

Serotype	"O" factors	"H" factors
1	I, II, (III)	a, b,
2	I, II, (III)	b, d,
3	II, (III) IV	a, b,
4a	(III), (V), VII, IX	a, b, c,
4b	(III), V, VI	a, b, c,
4c	(III), V, VII	a, b, c,
4d	(III), V, VI, VIII	a, b, c,
4e	(III), V, VI, VIII, IX	a, b, c,

Note: "O" factors III and V do not regularly occur. Factor (III) in various strains varies in its response to heat. Antibodies against this factor develop irregularly in immunized animals (5).

PREPARATION OF ANTIGENS

Known serotyping strains are inoculated onto tryptose agar plates and incubated at room temperature for 18 to 24 hours. The resultant growth is scraped off and suspended in phosphate buffered saline* (pH 7.2) washed by centrifugation and resuspended in the buffer. The suspension is divided into two aliquots, one of which is formalized ("HO" antigen), the other heated for one hour at 100° C in a boiling water bath ("O" antigen) and is preserved by the addition of 0.1% formalin. The suspensions are diluted to match Brown's or McFarland's tube No. 10. The "HO" antigen should also contain 0.1% formalin in the final dilution.

PRODUCTION OF ANTISERA

Only freshly prepared antigens should be used for the production of antisera. Healthy rabbits are intravenously injected at 4 day intervals with 0.5 to 1.0 ml. of antigen until a trial test titre of the rabbits serum against homologous antigen is 1:640 or over. The rabbit is then exsanguinated by heart puncture. The blood is allowed to clot at 4° C, serum separated and stored at 4° C until used. It is very difficult, even in the hands of an experienced worker, to serotype strains with unabsorbed antisera.

ABSORPTION TECHNIQUE

Serum to be absorbed is first titrated against the homologous strain as a reference. The antiserum is then 5 to 10% saturated with the absorbing strain and maintained at 45° C for two hours. The antigen is sedimented by centrifugation and the supernatant is again absorbed by the same technique. The abserbed antiserum is then titrated against the absorbing and the homologous strains. The only "H" factor necessary in the scheme is one containing factor "d". This factor is prepared by absorbing antiserum produced against a known serotype 2 "HO" antigen with serotype 1 "HO" antigen.

* Prepared in three litre lots as follows: 24 gm. sodium chloride, 238.5 ml. M/15 dipotassium phosphate, 61.5 ml. M/15 dihydrogen potassium phosphate q.s. to 3 litres with distilled water.

ABSORBING SCHEME FOR SPECIFIC "O" FACTORS

Serotype	Absorb with:	Specific factor(s)
1	Serotype 3 "O" antigen	I.
3	1 or 2 "O"	IV
4a	4c "O"	IX
4b	4a "O"	VI
4c	4b "O"	VII
4d	4b "O"	VIII
4e	3 "O"	V, VI, VIII, IX.

TECHNIQUE IN SEROTYPING

The known typing serum is diluted in a series of test tubes containing buffered saline solution giving dilutions of 1:10, 1:20, 1:40 etc. Unknown antigen, diluted to match Brown's tube No. 1, is added so that the volume of antigen is equal to the diluted antiserum. The tubes are shaken and incubated at 45° C in a water bath for 2 to 4 hours, refrigerated at 4° C overnight and the results recorded the following morning.

Agglutination reactions with patients sera should be interpreted with great caution due to cross reactions caused by antigens shared with certain other bacteria (7) and the high incidence of aggultinins versus serotype 1 listeria in normal sera (8). Therefore, the technique of serodiagnosis is not recommended unless executed by an investigator familiar with the difficulties that may be encountered. A rise or fall in titre may be regarded with some suspicion but *diagonisis rests solely on the isolation of the organism.*

NOTE

All organisms identified as *Listeria monocytogenes* should be forwarded to a central typing laboratory for serological classification. At the present time, to our knowledge, only two centers are equipped to serotype listeria strains, one in North America, the other in Europe. Strains should be lyophilized and sent either to:

Dr. H. P. R. Seeliger,
Listeria Typing Center,
Hygiene Institute, University of Bonn,
Bonn, Germany.

or

Dr. Wm. Cherry, Bacteriology Section,
Communicable Disease Center,
Atlanta, Georgia.

SUMMARY

A method is presented whereby *Listeria monocytogenes* may be isolated from biological specimens with relative ease employing the technique of Gray combined with a modification of McBride's Medium.

ACKNOWLEDGMENT

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